Transport properties of gastric $(H^+ + K^+)$-ATPase

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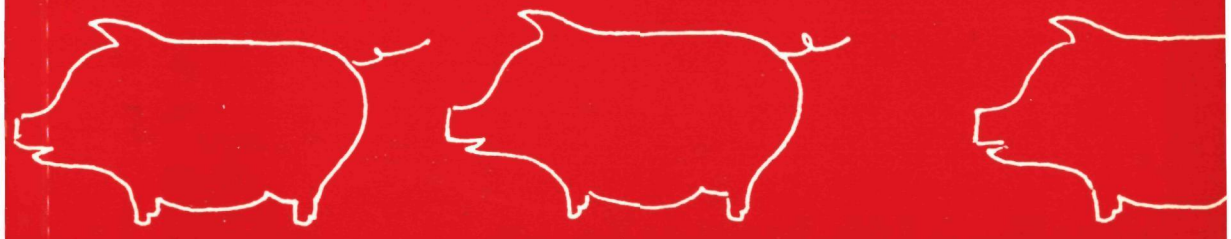

TRANSPORT PROPERTIES OF GASTRIC

$(H^+ + K^+)$-ATPase

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CONTENTS

CHAPTER ONE

GENERAL ASPECTS OF $(H^+ + K^+)$-ATPase

Introduction

A century ago, it was reported for the first time, that the acidity of the stomach was due to the secretion of hydrochloric acid (1). However, it was only recently, that it was possible to find the origin of this secretion, when a Mg^{2+}-dependent, H^+-transporting, K^+-stimulated adenosine triphosphatase was discovered in the parietal cell (2,3,4).

This so called (H^++K^+)-ATPase was then purified from the parietal cell of several different species as bullfrog (3), dog (4), rabbit (5), pig (6), chicken (7), rat (8) and man (9). It seems to be rather specific for this cell type, although recently a transport ATPase, that shows many resemblances, was identified in the distal colon of the rabbit, where it might be responsible for potassium uptake across the apical membrane (10).

The name (H^++K^+)-ATPase (E.C.3.6.1.36.) is worldwide used for this enzyme. However other names, such as (K,H)-ATPase and K^+-ATPase, have also been used in the past.

Purification

Generally $(H^+ + K^+)$-ATPase is purified by centrifugations of homogenized scrapings of gastric mucosa, resulting in a fraction of closed inside-out orientated membrane vesicles, the structure of which is revealed by electron microscopic examination. The intactness of these vesicles is also indicated by the stimulation of ATPase activity after addition of K^+-ionophores such as valinomycin and nigericin. The ionophore effect is lost after disruption of the vesicles (3,5).

The main activity is found in the fraction with a density of 1.12 g/ml. Upon further purification of the enzyme, leading to enhancement of the specific activity, the transporting capacity is lost, indicating that the purification procedure causes opening of the vesicles. Wolosin and Forte (11) gave evidence that the homogenization of stimulated gastric mucosa resulted in larger and denser membrane vesicles. This difference could be explained by morphological changes upon stimulation resulting in translocation of the enzyme from the tubulo-vesicular system to the apical plasma membrane (11). Larger vesicles could be the result of the aggregation of vesicles into canaliculi after stimulation of the parietal cell. The higher density would be the result of translocation of proteins and actin. The K^+-permeability of denser vesicles was much larger, suggesting that stimulation also modified the activity of the ionic transporters.

After SDS polyacrylamide gel electrophoresis of the purified membranes, one major protein band was found (12,13). This band had an apparent mass of 95-100 kD, close to the 110 kD mass, estimated by sedimentation analysis after solubilization with SDS (14). According to Saccomani et al (15), this band consists of three different proteins : a catalytic subunit, a glycoprotein and a third protein, resulting in a minimum molecular mass of 300 kD. The interpretation from these tryptic digestion studies was challenged by Peters et al (16), who found no evidence for heterogeneity of the subunits. Recent tryptic digestion studies by De Jong (17) do not support the concept of heterogeneity either. Very recently the primary structure of a catalytic subunit of rat gastric ATPase has been deduced from the cDNA structure (18). The molecular weight is 114,012 and there is 62 % homology with the α subunit of sheep kidney $(Na^+ + K^+)$-ATPase.

The isoelectric focussing of the detergent-solubilized protein gave more arguments for subunit heterogeneity, showing 3 peptides with different isoelectric points and distinct immunoreactivity (19). It could be that this

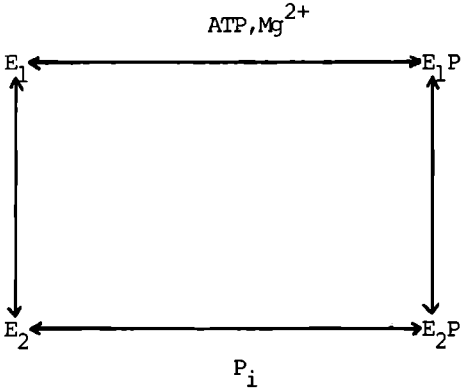
heterogenity is due to differences in the sugar composition of this band.

The molecular mass of the active $(H^+ + K^+)$-ATPase has not been established yet, since values of 270 kD (20) to 444 kD (21) have been found with radiation inactivation, whereas Soumarmon et al (22) found 350-390 kD with various separation techniques after n-octylglucoside solubilization. Therefore is it not yet possible to say whether the gastric $(H^+ + K^+)$-ATPase acts as a trimer or a tetramer of identical or different subunits. Rabon et al (23) recently proposed the enzyme to consist of a dimer of two identical parts, as suggested by the lattice parameters after crystallyzation of the enzyme., inactivated by vanadate.

In its purified state, the $(H^+ + K^+)$-ATPase is still surrounded by phospholipids. The phospholipid composition of this preparation is characteristic for a plasma membrane fraction, as also found for purified $(Na^+ + K^+)$-ATPase preparations (24). Schrijen et al (25) have investigated the phospholipid composition and the ratio of lipid per protein. The requirement of phospholipids for the activity of the enzyme was investigated by the use of phospholipases by Schrijen et al (26) and others (27). It was found that quantitatively the most important phospholipids were phosphatidylethanolamine, phosphatidylserine , phosphatidylcholine and sphingomyelin and that loss of 70 % of the activity was obtained after maximal hydrolysis of the phospholipids with two different phospholipases C, resulting in 70 % hydrolysis of the phospholipids.

Reaction mechanisms

For transport ATPases of this kind, a very simple scheme has been postulated. E_1 and E_1P indicate the forms with a high affinity for H^+ at the cytosolic side, E_2 and E_2P are forms with a high affinity for K^+, at the other side of the membrane.



During this cycle the enzyme is phosphorylated and dephosphorylated and the affinity for the ions varies in turn, leading to ion transport. Each cycle includes binding of ATP , phosphorylation and dephosphorylation. Each step will be discussed below.

* ATP binding

Several indications exist that binding of ATP induces a specific conformation of the enzyme, for example, it protects against tryptic digestion (15) and against the effects of side-specific reagents as diethyl pyrocarbonate (DEPC) (28), butanedione (29) and 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) (30). The fact that the rate of phosphorylation is higher when Mg^{2+} is added after mixing of the enzyme and ATP as compared to the rate after simultaneous mixing (31), also suggests that binding of ATP induces a specific conformation of the ATPase.

* Mg^{2+}-dependent phosphorylation

In the presence of Mg^{2+} the $(H^+ + K^+)$-ATPase is phosphorylated at an aspartyl-residue (32). The resulting phosphoenzyme is hydroxylamine-sensitive (33). Stimulation of the rate of phosphorylation is obtained when the pH at the cytosolic side is decreased (34). The phosphorylation is inhibited by the presence of K^+ at the cytosolic side (33,35) and low ATP/K^+ ratios make this step rate-limiting for the overall ATP hydrolysis (33). This phosphorylation step also occurs in the presence of Ca^{2+}, but is much slower (36). Perhaps a different phosphoenzyme is formed in that situation. Different

rate constants for the Mg^+-dependent phosphorylation are published, depending on pH (3650 per min at pH 7.4 (37) to 7900 per min at pH 6.0 (38)). This rate includes ATP binding plus enzyme phosphorylation : ATP binding was slower than the phosphorylation per se (37).

Helmich-De Jong et al (39) recently showed that a minor part of the phosphorylated intermediate is ADP sensitive. In adddition, they found that nucleotides inhibit the dephosphorylation rate through a low-affinity binding-site (17,40). Mg^{2+} again counteracts this inhibitory effect by lowering the affinity for nucleotides.

* Stoichiometry of ATP and phosphorylation sites

Phosphorylation with (γ^{32}P)ATP gives a maximal binding level of 1.6 nmol/mg protein (41), whereas with inorganic phosphate a level of 2.5-2.7 nmol/mg is found (42). With the ATP analogues AMPPNP (43) and N_3-ATP (44) nucleotide binding levels of 2.8 and 3.0 nmol/mg have been reported. Of the fluorescent probe FITC, which reacts with the ATP binding site in several transport ATPases (36), 1.5 nmol/mg was bound. With eosin (45), however, binding of 3.5 nmol/mg was found. The inhibitor vanadate showed the presence of two binding sites with different affinities each of which could bind 1.5 nmol/mg (41). These findings suggest the presence of 2 ATP binding sites per tetramer of 100-110 kD subunits. Of these sites only one can be phosphorylated by ATP.

* Dephosphorylation

When K^+ is added to the phosphoenzyme , a two-step dephosphorylation reaction is seen (37,46), an initial fast process with a rate constant of 4000 per min and a slower process with a rate constant of 270 per min, the latter not affected by K^+ concentrations larger than 0.5 mM. At low luminal pH, the overall hydrolysis of ATP could be limited by the K^+-dependent dephosphorylation (37).

Comparison

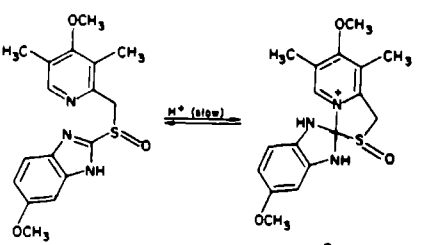
When this ATPase is compared with other ion-transporting ATPases, such as (Na^++K^+)-ATPase and Ca^{2+}-ATPase , many similarities are found , such as the size of the major subunit (100 kD), the elementary structure of this subunit (47-49) as well as its primary structure, the sensitivity to vanadate,

the occurrence of a phosphorylated intermediate and its K^+-stimulated hydrolysis (31,46,50-52). The recent molecular cloning of the rat stomach ATP ase (18) showed a striking similarity between this ATPase and the α subunit of sheep kidney (Na^++K^+)-ATPase in amino acid sequence and in hydropathy plot, suggesting that their higher order structure and mechanism of action are virtually identical.

Inhibitors

From a clinical point of view a specific inhibitor of the (H^++K^+)-ATPase could be helpful for inhibition of gastric secretion, to heal ulcera. From a biochemical point of view, such an inhibitor could be a useful tool for the estimation of the number of active enzyme molecules and for the elucidation of the reaction mechanism.

Untill now, the most promising inhibitor is omeprazole, a substituted benzimidazole.



It has been used in clinical studies, where it was shown to increase the rate of duodenal ulcer healing, and thus to be a potent drug in ulcer healing therapy (53). Unfortunately, it was also shown to develop gastric carcinoid tumors in rats, and for this reason, clinical experiments with omeprazole have temporarely been stopped (54). Wallmark et al (55) have shown that the weak base omeprazole (pKa=4) is protonated and accumulated in the acidic compartment of the parietal cell and chemically modified into an active inhibitor. The active inhibitor is a sulfonic acid or a sulfonamid, as has been shown by Lindberg et al (56). The active inhibitor is formed after protonation of the omeprazole, through a so-called spiro-intermediate.

The fact that the inhibition can be reversed by β-mercaptoethanol or dithiothreitol (55) indicates that the action of the omeprazole is due to the modification of SH-groups of the enzyme. Recently other inhibitors of $(H^+ + K^+)$-ATPase have been reported (SCH 32651 and SCH 28080), whose inhibition does not require an acidic activation and is not affected by the addition of sulfhydryl reducing agents (57), indicating that their mechanism of inhibition is different from that of omeprazole.

Transport capacity of $(H^+ + K^+)$-ATPase

The $(H^+ + K^+)$-ATPase is able to exchange H^+ for K^+ across the plasma membrane upon hydrolysis of ATP. The transport of protons can be studied using native gastric membrane vesicles, as was first described by Lee et al (4) in 1974. The native vesicles were used to study the H^+/ATP ratio of the transport process. The most direct way to do this, was by measuring the disappearance of protons from the extravesicular medium with a pH electrode at different concentrations of ATP. However, this method can only be used at pH 6.1, because otherwise ATP hydrolysis itself induces pH variations (58). With pH electrode measurements, different H^+/ATP ratios have been found, varying from 4 to 1 (12,59-61).

Transport of K^+ has been studied with ^{86}Rb as tracer. Using this tool, it was demonstrated that in the presence of ATP, H^+ is exchanged for K^+. In the absence of ATP, a slow K^+ diffusion pathway and a fast K^+:K^+ exchange were observed (62,63). K^+:K^+ exchange could be inhibited by nucleotides or vanadate, suggesting that $(H^+ + K^+)$-ATPase was involved (63). This K^+:K^+ exchange has also been described for reconstituted ATPase (64).

A K^+-occlusion step that would be rate-limiting, as has been described for the $(Na^+ + K^+)$-ATPase (65,66), has not been found for the $(H^+ + K^+)$-ATPase, because the rate of maximal passive K^+ exchange was nearly as high as that of Rb^+ efflux after ATP addition.

The impurities still present in the enzyme preparation and the presence of damaged vesicles may be a problem when kinetic or transport studies are carried out. Therefore reconstitution is a great help for studies of $(H^+ + K^+)$-ATPase.

Reconstitution

Natural vesicles have been used to study transport mechanisms, but both impurity of the preparation and rapid saturation of transport kinetics made it desirable to do transport studies with reconstituted enzyme. Reconstitution is in fact the incorporation of a protein in a well-known phospholipid bilayer system with the purpose to investigate the properties of the protein. In our case we wanted to incorporate (H^++K^+)-ATPase into vesicles in order to measure the transport capacities of the enzyme. To do this unilamellar vesicles were required with significant intravesicular volume, to overcome the difficulties of the rapid saturation of transport kinetics of the natural membrane vesicles.

To reach such a point several steps have to be taken, such as the solubilization of the protein in an active form, the preparation of bilayer membranes with a specific phospholipid composition and the incorporation of the protein in the lipid system. The resulting protein-containing vesicles are called proteoliposomes. The average diameter of proteoliposomes depends on the method used for their preparation : sonication of lipids and protein gives small vesicles,whereas dispersion with detergents followed by dialysis gives larger vesicles. In case of a transport protein like (H^++K^+)-ATPase, ion transport is the parameter of choice to be investigated.

Reconstitution consists in most cases of the solubilization of the protein, the addition of phospholipids and the removal of the excess detergent. Several methods have been applied for detergent removal. Detergent-dialysis has been used by Goldin (67), who reconstituted purified (Na^++K^+)-ATPase with the use of cholate, by simultanuous mixing of enzyme, detergent and phospholipids. This reconstitution method is followed by a dialysis step, which eliminates the detergent. This technique requires the use of a dialysable detergent such as cholate or n-octylglucoside, but prevents the use of Triton X-100, whose micelles are too large.

Disadvantage of the dialysis method is the long duration (10 to 48 hours) of the process. A faster method is detergent-dilution as has been applied by Racker et al (68,69), After protein-phospholipid-detergent mixing, the medium is diluted in order to bring the detergent concentration under its critical micellar concentration (cmc). A disadvantage of this method is the low protein concentration after the dilution. However this technique is less successful than dialysis in the percentage of protein reconstituted. To optimalize the reconstitution, sonication of the mixture of protein and phospholipids was

introduced by Racker (70). This method was, even without detergent successful for reconstitution of Ca^{2+}-ATPase. Later a freeze-thaw step was used prior to sonication, which made sonication time shorter and reduced the chance on inactivation (71-73).

Eytan et al (74,75) incorporated the protein in phospholipid liposomes (made by sonication), only by incubation at room temperature, eventually in the presence of a detergent. This method avoided sonication, so that the chance on denaturation was diminished. However, the yield of reconstitution was very small.

Solubilization and reconstitution of $(H^+ + K^+)$-ATPase

To reconstitute purified $(H^+ + K^+)$-ATPase, a solubilization step with a detergent is desirable, as shown above.

It was already known, that sodium dodecyl sulfate (SDS) could be used to extract a 100 kD protein from a membrane fraction (76), but the activity was then completely lost. Using the mild detergent n-octyl-glucoside, Soumarmon et al (22) succeeded for the first time to solubilize an active form, which could not be pelleted by centrifugation during one hour at 100,000 g. They recovered 37 % of the $(H^+ + K^+)$-ATPase activity, and up to 70 % of the protein was found in the supernatant. When the detergent was diluted or dialyzed, the activity of the enzyme was restored, but not to the starting level, indicating denaturation of the protein by n-octylglucoside.

Later it was shown that the solubilization of the native polymer occurred at the same time as it depolymerized. Depolymerization was suggested to convert the enzyme into an inactive monomer. Three intermediate forms were described (77). Instead of n-octylglucoside, it was also possible to use cholate, or a combination of the two detergents in a two-step solubilization, to obtain more active enzyme.

These experiments, in which $(H^+ + K^+)$-ATPase was solubilized in an active form, were the starting point for the reconstitution of the enzyme in liposomes. The method used for the reconstitution of the $(H^+ + K^+)$-ATPase is a freeze-thaw cycle followed by sonication . This method was first used by Rabon et al (58), who reconstituted active $(H^+ + K^+)$-ATPase into phosphatidylcholine/ cholesterol liposomes and measured ion transport by the enzyme in this system.

We learned this method, as described in chapter 4, during a stay at the

laboratory of Dr. Lewin and Dr. Soumarmon (INSERM U10,Paris), and made it a useful tool for the studies of K^+:H^+ exchange of the gastric $(H^+ + K^+)$-ATPase.

Content of this thesis

In this thesis experiments are described, which were carried out with a $(H^+ + K^+)$-ATPase preparation from pig gastric mucosa. In previous studies of our laboratory, several aspects of the reaction mechanism of $(H^+ + K^+)$-ATPase have been studied (17,25). In this thesis the transport properties of this enzyme have been studied. Purpose was to investigate the ion transport ratios of the enzyme and to reconstitute the $(H^+ + K^+)$-ATPase in well defined liposomes.

Chapter 2 describes the determination of the H^+/ATP transport ratio in native gastric membrane vesicles with the use of a pH meter technique.

In chapter 3 experimental data are given on the solubilization of purified $(H^+ + K^+)$-ATPase with n-octylglucoside. The properties of the solubilized form of the enzyme have been studied. In chapter 4 the reconstitution procedure is described and optimalization of the techniques is discussed.

Chapter 5 deals with the passive and active H^+-transport by reconstituted $(H^+ + K^+)$-ATPase, monitored with pH measurements. Effects of inhibition are also shown.

Chapter 6 includes more data about transport by reconstituted enzyme; here mainly Rb^+ transport is discussed. In chapter 7 the ratio between the number of transported ions and ATP hydrolysed is determined for reconstituted $(H^+ + K^+)$-ATPase after separation of proteoliposomes and the non-incorporated protein.

In chapter 8 the results are summarized and discussed in a wider perspective.

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CHAPTER TWO

THE H^+/ATP TRANSPORT RATIO OF THE (H^++K^+)-ATPase OF PIG GASTRIC MEMBRANE VESICLES

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slightly modified from
Biochim. Biophys. Acta 774, 91-95 (1984)

SUMMARY

Various values have been reported for the H^+/ATP transport ratio of the (H^++K^+)-ATPase of the gastric parietal cell : 4,2 and 1. We have, therefore, reinvestigated this matter with a vesicle preparation isolated from pig gastric mucosa. The vesicles are suspended in glycylglycine buffer (pH 6.11) at 22°C, and incubated until equilization of the K^+ concentration inside and outside (75 mM). After addition of ATP, the initial rates of H^+ uptake and ATP hydrolysis are then measured. Proton uptake is inhibited in the absence of K^+ or in the presence of nigericin. The $K_{0.5}$ value for proton transport is 154 µM and the K_m value for ATP hydrolysis is 61 µM. The Lineweaver-Burk plot for ATP hydrolysis versus ATP concentration is linear with a V_{max} of 5.5 nmoles per mg protein per sec, but that for H^+ uptake is not. Thus with increasing ATP concentration (6.7 to 1670 uM) the transport ratio increases from 0.3 to 1.8. Extrapolation to infinite ATP concentration gives a value of 1.89 (SE=0.13,n=5) and a Hill coefficient of n=1.21 (SE=0.06,n=5) implying that the true transport ratio is 2 H^+/ATP with positive cooperativity between the protons.

INTRODUCTION

Resting gastric parietal cells contain a multitude of tubular vesicles, which upon isolation have been shown to possess a high activity of a K^+-stimulated ATPase. This enzyme, usually called $(H^+ + K^+)$-ATPase, has been shown to be responsible for proton uptake into the vesicles at the expenditure of ATP (1,2). This process has been demonstrated in vesicles from pig (3,4) and dog (5,6) gastric mucosa.

Divergent values for the H^+/ATP transport ratio have been reported: 4 by Sachs et al (7) in 1976, 2 by Rabon, McFall and Sachs (3) in 1982, and 1 by Reenstra and Forte (4) in 1981 and by Smith and Scholes (6) in 1982. Thermodynamically, a value of 4 would be unlikely in view of the 10^5:1 gradient which can be generated in the stomach. The other two values would both be thermodynamically possible.

From a study of the conditions used by these investigators, it became clear that in all but one study fixed ATP concentrations have been used. This has led us to an investigation in which a wide range of ATP concentrations has been used. In addition we have determined some kinetic parameters of H^+-transport and ATP hydrolysis, and we have investigated the cooperativity of H^+-transport.

METHODS

Preparation of gastric membrane vesicles

Isolation of the gastric vesicles has been carried out according to the procedure previously reported by Forte et al (8,9,10), Sachs et al (7,11,12) and Schrijen et al (13) with some modifications.

Stomachs from freshly slaughtered pigs are obtained from the local slaughterhouse and after removal of the contents transported to the laboratory on ice. All subsequent manipulations are carried out at $4^{\circ}C$. The stomachs are rinsed with tap water and the fundic region is taken and placed in 3 M NaCl. Mucus is removed by wiping the tissue with paper towels and the mucosa is scraped from the underlying muscular layer by means of a surgical blade. The scraped material (70-80 g) is placed in 200 ml homogenization buffer, containing 113 mM mannitol, 37 mM sucrose, 0.2 mM EDTA, 5 mM Tris-HCl (pH 7.8). The mixture is homogenized with a Braun teflon-glass homogenizer by

three up-down strokes of the rotating pestle (250 rev/min).

The homogenate is filtered over four layers of surgical gauze and centrifuged for 20 min at 20,000 g in a Sorvall RC 2-B centrifuge (GSA rotor,4°C). The supernatant is centrifuged for 60 min at 100,000 g in a MSE Prepspin 50 centrifuge (8x50 rotor,4°C). The resulting pellet is resuspended in 20 ml homogenization buffer. The suspension is layered on top of a discontinuous gradient of 20 ml 20 % (w/v) sucrose and 10 ml 37 % (w/v) sucrose. The gradient is centrifuged for 40 min at 80,000 g in a MSE Europe 75 centrifuge (rotor TST 28-38,4°C).

The vesicle fraction at the interface of supernatant and 20 % sucrose layer is collected by suction. It is diluted 1:1 with a buffer containing 5 mM glycylglycine, 150 mM KCl and 2 mM $MgCl_2$ (5), and the pH is adjusted to 6.05-6.10. The resulting suspension is stored overnight at 4°C to equilibrate the vesicles with respect to pH and K^+ content. The next day this fraction is used for measurements of H^+-transport and ATP hydrolysis. The fraction at the interface of the 20 % and 37 % sucrose layers can be collected for the purification of $(H^+ + K^+)$-ATPase by centrifugation on a continuous sucrose gradient according to Schrijen et al (13).

Proton uptake measurements

The uptake of protons from the extravesicular medium is measured at 22 °C by recording the change in the medium pH after addition of ATP (3). Four ml of gastric membrane vesicles (4-6 mg protein), equilibrated overnight at 4°C in glycylglycine buffer, are placed in a small glass vessel. The suspension is adjusted to pH 6.11 and is magnetically stirred throughout the experiment, while the pH is continuously recorded with a Radiometer GK 2321C combined electrode connected to a Radiometer PHM 75 Research pH meter with a BD 40/60 recorder (Kipp and Sons,Delft).

Measurements are started by adding kown volumes of a 5 mM MgATP solution, adjusted to pH 6.11. Any pH changes due to the addition of ATP are corrected for. Subsequently, the proton uptake is determined by titrating the suspension with 1 mM KOH or 1 mM HCl. The response time of the electrode is negligible (less than 2 sec).

Valinomycin and nigericin are added as 1 mg/ml solutions in methanol.

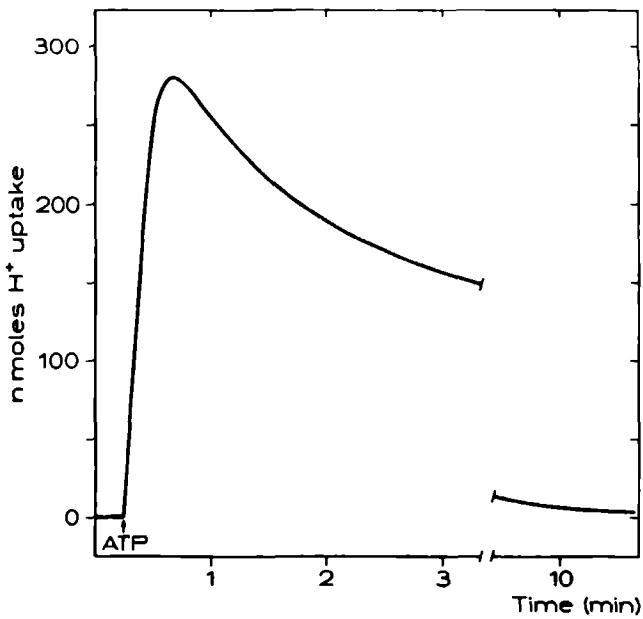

Figure 1

Proton transport in gastric parietal cell vesicles

Typical experiment with 5.2 mg protein in 4 ml vesicle suspension, addition of 2 mmol MgATP, final concentration 455 uM. Reproduction of pH meter recording.

ATP hydrolysis measurement

During proton uptake measurements 100 µl aliquots are taken from the vessel for phosphate determination according to a modification of the procedure of Carter and Karl (14). The aliqouts are added to 1 ml of a solution containing 0.85 M HCl, 0.3 % Na-molybdate, 0.2 % Bion-Ne-9 to stop the enzyme reaction. Then 320 µl malachite green solution (6.3 mg/100 ml) is added, and immediately after mixing the extinction at 625 nm is read and compared with phosphate standards similarly treated.

Protein determination

The method of Lowry et al (15) is employed with TCA precipitation of the protein and bovine serum albumin as standard.

Chemicals

MgATP, nigericin and valinomycin have been purchased from Sigma (St Louis,MO,USA), bovine serum albumin from Behringwerke (Marburg, FRG), Bion-Ne-9 from Pierce (Rockford,IL,USA), malachite green from Aldrich (Milwaukee,TN,USA) and sucrose from Janssen (Beerse,Belgium).

All other chemicals were purchased from Merck (Darmstadt,FRG).

RESULTS

Centrifugation on the discontinuous sucrose gradient (37 %, 20 % sucrose,supernatant) yields two vesicle fractions: one at the 20/37% interface, and one at the supernatant/20% sucrose interface.

The $(H^+ + K^+)$-ATPase activity in the first fraction is 40 µmoles P_i per mg protein per h when measured at 37°C, pH 7.4 in the presence of 20 mM K^+ and 5 mM ATP and after correction for other ATPase activity by replacing K^+ with 20 mM choline chloride. The activity is not stimulated by valinomycin addition and the vesicles do not exhibit proton uptake. So these vesicles are presumably leaky, and they have not been used in any of the experiments reported below. The supernatant/20 % sucrose interface fraction has a $(H^+ + K^+)$-ATPase activity of 12 µmoles per mg per h, when determined under the same conditions. This activity can be stimulated 80-100 % of by the

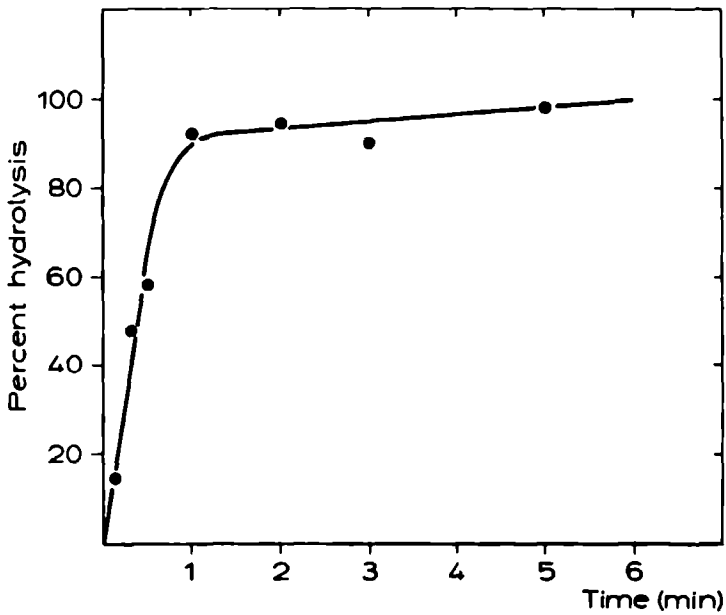

Figure 2

ATP hydrolysis by gastric parietal cell vesicles.

Typical experiment with 3 mg protein in 4 ml vesicle suspension. ATP hydrolysis is plotted versus time following addition of 100 nmoles MgATP.

addition of 10 µl valinomycin (1 mg/ml in ethanol), and the vesicles in this fraction exhibit H^+ uptake capacity. This fraction is assumed to consist of closed vesicles and it has therefore been used in all further experiments.

A typical curve for proton uptake is shown in fig. 1. Proton uptake reaches a maximum within 30 sec after addition of ATP. Afterwards passive efflux of protons dominates and the medium pH returns to the initial value after 5-10 min. The proton efflux can be enhanced by addition of the K^+-ionophores valinomycin and nigericin, in which case the pH returns within 90 sec. Addition of nigericin before ATP removes the ability of the vesicles to accumulate protons, so that no pH change occurs upon addition of ATP. Addition of valinomycin before ATP does not have this effect, because there is no K^+ gradient between the equilibrated vesicles and the buffer medium. However, addition of valiomycin after ATP addition, when the uptake of H^+ has caused an equivalent extrusion of K^+, causes K^+ influx in exchange for H^+, thus speeding up the return of the medium pH. From the plot of phosphate release versus time (fig. 2), we can calculate that the maximal medium pH is reached when 80-90 % of the added 100 nmol ATP has been consumed. When the vesicles are equilibrated in choline chloride, the ATPase activity is only about 5 % of the activity after equilibration with KCl, and the proton transport is negligible. When the vesicles are washed to remove the last traces of K^+, and then equilibrated with choline chloride, the ATPase activity is only 1 % and proton transport is zero.

The rates of H^+-transport and ATP hydrolysis during the first 10 sec are constant, and have therefore been used in determining the H^+/ATP transport ratio. This ratio has been determined at different ATP concentrations in the following way. Lineweaver-Burk plots have been constructed for the rates of ATP hydrolysis and H^+ transport in the initial 10 sec period against the average ATP concentration (ATP concentration at 0 sec plus that at 10 sec, divided by 2). Fig. 3 gives a typical example of such Lineweaver-Burk plots. The two plots are not parallel, which has been consistently found in all experiments. We conclude that the transport ratio increases with the ATP concentration to a maximal value of nearly 2 at infinite ATP concentration.

Table I summarizes the results of 5 experiments. The average transport parameters for proton transport are V_{max}=10.2 nmoles H^+ per mg per sec (SE=0.3) and $K_{0.5}$= 154 µM (SE=5), and for ATPase activity V_{max}= 5.5 nmoles P_i per mg per sec (SE=0.3) and K_m= 61 µM (SE=7). The average H^+/ATP transport ratio at infinite ATP concentration is 1.89 (SE=0.13), which value is signifficantly higher than 1 and not signifficantly different from 2. The

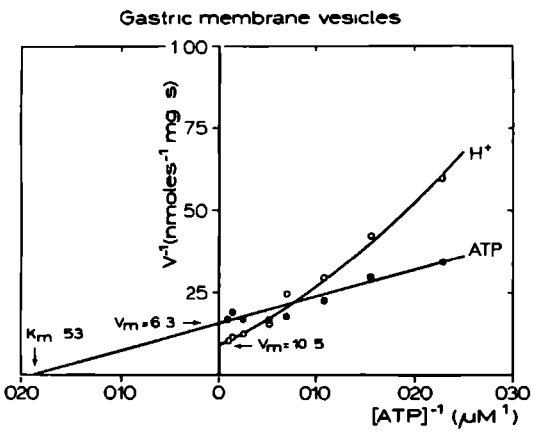

Figure 3

Lineweaver-Burk plot of transport rate and ATP hydrolysis rate versus ATP concentration.

The straight line through the experimental datapoints for ATP hydrolysis has been determined by linear regression analysis. K_m and V_{max} values are determined as the intercepts on the abscissa and ordinate. The best fitting curve through the experimental points of H^+-transport is drawn visually, and V_{max} is extrapolated as intercept on the abscissa. All points have been determined with 5.2 mg protein in 4 ml vesicle suspension.

curved Lineweaver-Burk plot suggests the presence of an allosteric effect in proton transport. Hill plots constructed from these data are linear and yield a Hill coefficient of 1.21 (SE=0.06), which is significantly higher than 1 and thus confirms positive cooperativity in proton transport.

DISCUSSION

We find a transport ratio increasing from 0.4 at 8 um ATP to a maximum of 1.89 at infinite ATP concentration. This may explain the different ratios reported by Lee and Forte (4) and Smith and Scholes (6), who found a ratio of 1, and by Rabon et al (7), who observed a ratio of 2. Smith and Scholes used a low concentration of 50 µm ATP, while Rabon used four different concentrations giving a wide spread of ratios, up to a ratio of 2 for the highest concentration of 255 µm ATP. In view of our results, we may conclude that the different transport ratios of Smith and Scholes and Rabon et al are due to the different ATP concentrations used. It does not explain the low transport rat io of 1 reported by Lee and Forte, for they used a high concentration of ATP (1000 uM), which in our experiments gives a ratio signifficantly greater than 1. Their low ratio might be due to to their way of preparing and storing the vesicles, particlarly the storage of the material as a frozen suspension. The freezing of the material could very well damage the integrity of the vesicles, leading to a high outward leakage of protons during proton uptake.

Our maximal transport ratio of 1.89 could be somewhat low for two reasons. First the total proton uptake might be underestimated because of the contribution of protons leaking out during the first 10 sec of measurement, which would result in a somewhat lower peak in the plot of pH versus time. Secondly a small part of the amount of hydrolyzed ATP could be due to the presence of leaky vesicles, which are able to hydrolyze ATP, but not to transport protons. It is unlikely that the presence of other ATPases would lower the ratio, since in the absence of Na^+ and Ca^{2+} neither $(Na^+ + K^+)$-ATPase, nor Ca^{2+}-ATPase could be active.

Thus we assume a true transport ratio of 2 protons per ATP. Thermodynamically this ratio is the highest possible, as shown by the following calculations. A pH of 4.5 units is found to be maximally generated by gastric membrane vesicles (17). The equation for the needed energy is as follows :

TABLE I

RESULTS FOR PROTON TRANSPORT BY GASTRIC MEMBRANE VESICLES

V_{max} for H^+ transport (nmoles $mg^{-1}sec^{-1}$)	V_{max} for ATPase activity (nmoles $mg^{-1}sec^{-1}$)	H^+/ATP	Hill coefficient
10.0	4.6	2.16	1.40
10.0	5.9	1.69	1.19
11.1	4.8	2.31	1.06
10.5	6.3	1.67	1.30
9.5	5.7	1.68	1.08
10.2 (SE=0.3)	5.5 (SE=0.3)	1.89 (SE=0.13)	1.21 (SE=0.06)

$K_{0.5}$=154 ± 5 μM K_m=61 ± 7 μM

$$G = R\ T\ \ln \frac{c_2}{c_1} = R\ T\ \ln\ 10^{4.5} = 6.0\ \text{kcal/mol}$$

If two protons are transported, this would thus require 12 kcal. The hydrolysis of ATP has a free energy of - 13.4 kcal/mol, which is sufficient for the transport of 2 H^+/ATP, and in addition transport of K^+ against a gradient, which is necessary when a electroneutral pump is assumed (17).

The curved shape of the Lineweaver-Burk plot for H^+ uptake and the Hill coefficient of 1.21 (derived from plotting log v/v_{max}-v versus log [ATP]), indicate positive cooperativity of two proton sites involved in proton transport. The curved shape of the proton uptake plot explains why the H^+/ATP transport ratio increases at increasing ATP concentration. The Hill coefficient larger than 1 indicates that the transport of one proton facilitates the transport of a second proton. The low ratios at low ATP concentration are probably due to the increase of proton leakage from the vesicles relative to the active proton uptake.

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CHAPTER THREE

PROPERTIES OF $(H^+ + K^+)$-ATPase SOLUBILIZED BY n-OCTYLGLUCOSIDE

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SUMMARY

After purification of the $(H^+ + K^+)$-ATPase, a membrane bound enzyme is obtained, embedded in a phospholipid surrounding of known composition (Schrijen et al ,1981, Biochim. Biophys. Acta 649, 1-12). These phospholipids are necessary for the function of the $(H^+ + K^+)$-ATPase.

Solubilization of the enzyme, a step necessary for reconstitution, can be performed with n-octylglucoside. Under optimal conditions 24% of the activity can be found in the supernatant after centrifugation of the detergent/protein mixture. Upon dilution 90% of the activity is restored. The properties of the solubilized enzyme, like phosphorylation, K_m for ATP and K_m for K^+ are not different from the native enzyme. The amount of phospholipids bound to the enzyme is significantly reduced. A minor part of the detergent is firmly bound to the enzyme.

INTRODUCTION

After determination of the H^+/ATP ratio of gastric membrane vesicles (1), we wanted to investigate the transport properties of more purified enzyme preparations and to determine the transport ratio of those preparations, since the determined ratio of 2 could be an underestimate because of the presence of "leaky" vesicles and other proteins.

Since purified enzyme preparations had lost their transport ability, it was necessary to reconstitute the ATPase into artificial vesicles to restore the function. In most reconstitution procedures solubilization with a detergent is a necessary step. We therefore studied the effect of a mild detergent, n-octylglucoside, on (H^++K^+)-ATPase. In particular we were interested in the effect of the detergent on the properties and composition of the enzyme preparation.

We succeeded to solubilize 54 % of the protein and 24 % of the ATPase activity at a detergent concentration of 15 mg/ml and a protein/ detergent ratio of 1. Only a small part of the detergent is bound to the enzyme, and the amount of lipid environment of the solubilized protein is significantly different from that of the native enzyme.

METHODS

Preparation of membrane fraction

Stomachs of freshly slaughtered pigs were transported to the laboratory on ice. After flushing with tap water and cleaning with paper towels, the mucosa of the fundic region was scraped off and homogenized in a buffer containing 150 mM sucrose, 0.2 mM EDTA and 5 mM Tris-HCl (pH 7.2). After three up-down strokes of the rotating pestle (1000 rev/min) of a Braun teflon-glass homogenizer, the mixture was centrifuged for 20 min at 20,000 g (Sorvall,GSA rotor). The resulting supernatant was centrifuged 45 min at 100,000 g (MSE,8x50 rotor) and the pellet of this step was resuspended in 25 mM Tris-HCl (pH 7.4).

The resuspended pellet was centrifuged on top of a gradient consisting of 7 % Ficoll/250 mM sucrose in 25 mM Tris-HCl over 37 % sucrose in 25 mM Tris-HCl. After 60 min centrifugation at 100,000 g (MSE,8x50), the interfase was diluted in 25 mM Tris-HCl and centrifuged for 60 min at 120,000 g

(MSE,10x10 rotor).

The final pellet was resuspended in a small volume of 25 mM Tris-HCl and frozen at -30°C. The specific activity of the enzyme preparations ranged from 70-90 μmoles per mg per hour.

Solubilization

Solubilization with n-octylglucoside was carried out as follows. Membrane preparations of 10-15 mg/ml in 250 mM sucrose, 2 mM dithiothreitol and 50 mM Hepes-Tris (pH=7.2) were treated with n-octylglucoside in a final concentration of 15 mg/ml. After incubation at 4°C for 15 min, the mixture was centrifuged for 60 min at 100.000 g (Beckman,Ti 50 rotor). The solubilized protein was found in the supernatant of this step. The pellet was resuspended in the same buffer. This method is nearly the same as used by Soumarmon et al (2), who were the first to publish the solubilization of an active form of $(H^+ + K^+)$-ATPase.

ATPase assay

K^+-ATPase activity of the enzyme was measured in 30 mM imidazole-HCl (pH 7.5), 5 mM Mg^{2+}, 0.1 mM ouabain, 20 mM KCl or 20 mM choline chloride and 5 mM Na_2ATP. To 400 ul of this medium, 20 ul of enzyme suspension was added after which incubation took place for 10-30 min at 37°C. Stopping of the reaction was done by adding 3 ml of a 1 : 1 mixture of 9.2 % (w/v) Fe_2SO_4 in 0.66 M H_2SO_4 + 1.15 % ammoniummolybdate and 8.6 % trichloracetic acid. After 30 min the extinction at 700 nm was read and compared with the extinction of a standard treated in the same way.

Protein measurement

Protein was determined with the method of Lowry et al (3) or fluorimetrically using an excitation wavelength of 278 nm and an emission wavelength of 340 nm. In both cases bovine serum albumin was used as standard.

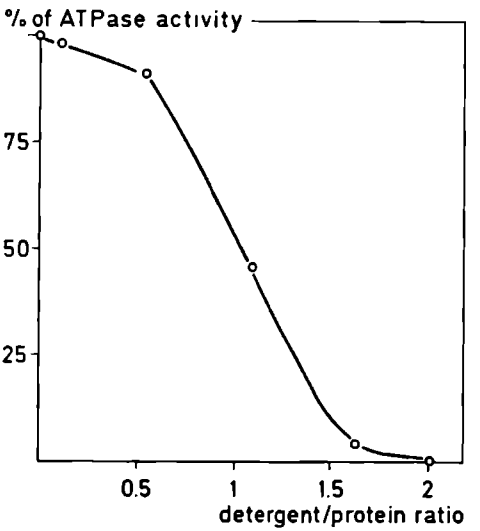

Figure 1

Influence of the detergent/protein ratio on ATPase activity.

Protein 9.3 mg/ml, incubation time with n-octylglucoside was 30 min on ice; ATPase determination for 10 min at 37°C in the detergent/protein mixture. Representative for 6 experiments.

Radioactive ATP hydrolysis measurement

50 µl aliquots of enzyme were added to 150 µl of a medium containing 95 mM Tris-HCl, 16 mM $MgSO_4$, 0.3 mM ouabain, 0.3 mM EDTA and 32 mM choline chloride or 16 mM K_2SO_4; 200 µl of a stock solution containing 0.87 mM ATP and tracer γ ^{32}P-ATP was then added and the mixture was incubated at room temperature.

The reaction was stopped by adding 800 µl 5 % TCA/10 % Norit (w/v) After 10 min the mixture was vortexed again, and centrifuged for 10 min at 5000 g (Hereaus Christ). 500 µl of the supernatant was taken and counted in a Philips Liquid Scintillation Analyzer.

Lipid analysis and phosphate determination

Lipids were extracted with the method of Folch et al (4), with the modification, that dichloromethane was used as solvent instead of chloroform.

Phosphate content of membrane samples were determined with the method of Fiske and SubbaRow (5). Samples were digested with 0.2 ml concentrated H_2SO_4/ $HClO_4$ for 1 hour at 180^{o}C. The tubes were then cooled below 50^{o}C. In case the destruction was incomplete, 0.1 ml 30 % H_2O_2 was added to the tubes and the destruction continued for at least 15 min, untill the samples were colourless. After cooling, 4.75 ml of colour reagent was added, consisting of a freshly prepared mixture of 50 ml of a solution containing 2.60 g $(NH_4)_6Mo_7O_{24}.4H_2O$ and 2.2 ml of a solution containing 30.1 mg $Na_2S_2O_3$ + 11 mg $Na_2S_2O_3$ + 55 mg aminonaphtalene sulphonic acid, according to Broekhuyse (6).

The contents of each tube were mixed and incubated for 20 min in a boiling water bath. After cooling with tap water, the 820 nm absorbance was measured against water. In each determination a series of standard P_i samples was included and similarly treated.

Binding studies

Purified (H^++K^+)-ATPase was mixed with a stock solution of n-octylglucoside, containing ^{14}C-labeled n-octylglucoside and eluted over a C18 affinity column (Waters Associates), that was activated with ethanol, to

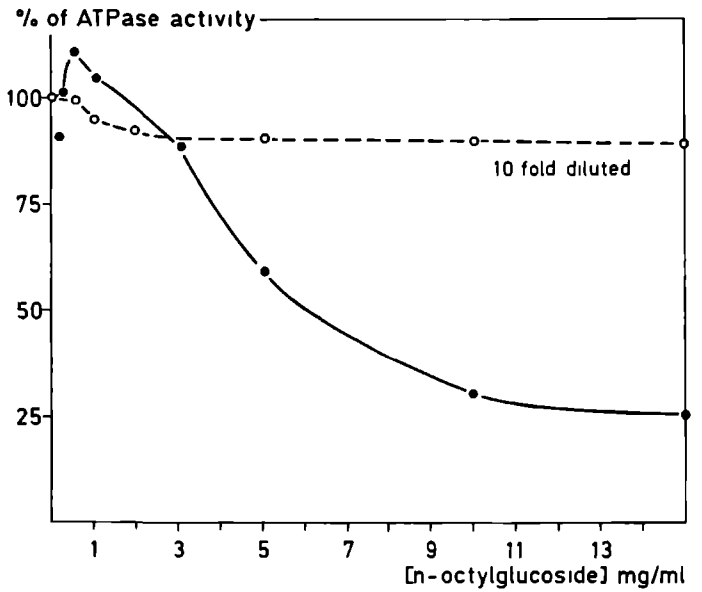

Figure 2

Influence of the concentration of n-octylglucoside on ATPase activity at a detergent/protein ratio of 1.

The detergent/protein mixture was centrifuged for 60 min at 100,000 g and ATPase activity of the supernatant was determined, as well as activity of the supernatant diluted 10 times in detergent-free buffer. The assay was carried out at 37°C for 15 min and 30 min, respectively. In the control the $(H^+ + K^+)$-ATPase activity was 78.6 umoles/mg.h. Representative for 4 experiments.

remove non-bound detergent. The resulting eluate was counted in a Philips Liquid Scintillation Analyzer.

Gradient centrifugation

For lipid separation, a discontinuous gradient was used of 1 ml 45 %, 3 ml of 20 % and 3 ml of 10 % sucrose in and 25 mM Tris-HCl (pH 7.5). On top of the gradient 400 ul sample of the solubilized enzyme was brought. Centrifugation was carried out for 1 hour at 100,000 g in a Ti 50 rotor (Beckman centrifuge). Fractions of 200 ul were collected.

Chemicals

MgATP was purchased from Sigma (St Louis,MO,USA) and γ^{32}P-ATP and ^{14}C-n-octylglucoside from Amersham (UK).

All other chemicals were purchased from Merck (Darmstadt,FRG), except Ficoll, which was from from Pharmacia Fine Chemicals (Uppsala,Sweden) and sucrose, which was from from Janssen (Beerse,Belgium).

RESULTS

Our first solubilization experiments were carried out with n-octylglucoside, a non-ionic detergent, with a critical micellar concentration (cmc) of 25 mM. This had been used in the same time by Soumarmon et al (2) to solubilize the (H^++K^+)-ATPase in an active form. With increasing detergent concentrations, increasing quantities of protein were found in the supernatant after centrifugation of the detergent/protein mixture, as shown in Table 1. The best results were obtained using a n-octylglucoside concentration of 15 mg/ml (this concentration gives a better activity recovery than 30 mg/ml) and a detergent/protein ratio of 1, as found earlier by Soumarmon et al (2).

However, the solubilized protein had 20-30% of its initial ATPase activity. This inactivation depended upon the ratio of protein to detergent as shown in fig. 1 and also on the concentration of detergent (at fixed protein/detergent ratio) as is shown in fig. 2. At higher concentrations, more inactivation occurred. When the solubilized protein was diluted 10 times

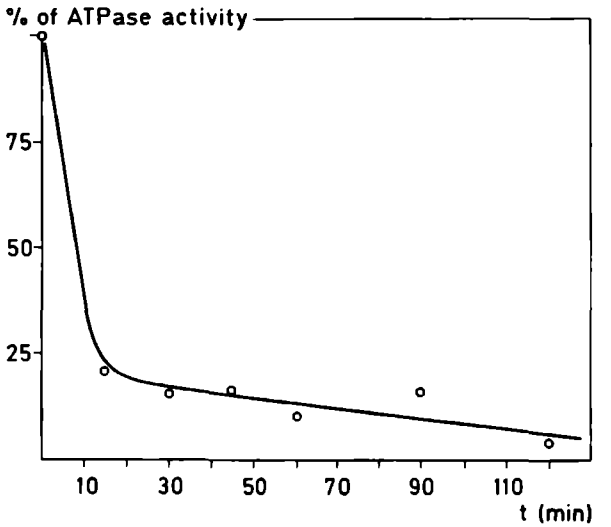

Figure 3
Time curve of inactivation of $(H^+ + K^+)$-ATPase by n-octylglucoside. Concentration of detergent was 50 mM, protein was 14.4 mg/ml, incubation was on ice. The ATPase assay was carried out at 37°C for 10 min with the detergent/protein mixture.

before the ATPase activity was measured, the activity was nearly the same at all detergent concentrations i.e 90% of the initial activity, indicating that part of the inactivation was reversible . In fig. 3 inhibition is shown to be time-dependent, with a fast decrease in the first 15 min, followed by a slower decrease in the next period.

The properties of the solubilized $(H^+ + K^+)$-ATPase and of the non-solubilized form were compared (Table 2). Specific activity of the ATPase activity was 83% decreased after detergent treatment, whereas the phosphorylation level was decreased to about 11 % of the level of the native enzyme (n=2). Affinities of the enzyme for ATP and K^+ were not changed. Only a part of the phospholipid content was lost after the treatment with detergent. These results suggest that the turnover rate of the $(H^+ + K^+)$-ATPase as well as its capacity to be phosphorylated are affected when the protein is solubilized with n-octylglucoside but that the mechanism of ATP hydrolysis remains the same.

Under standard experimental conditions, 3.5 nmol of n-octylglucoside were associated to one mg of protein.This represented 1.5 % of the total detergent used. This value is comparable to that of ATP binding (3.0 nmol per mg protein)(7-9), and corresponds to approximately 2 sites per tetramer of 100 kD subunits.

Separation of the solubilized enzyme and the phospholipids was attempted using a Sephadex G 50 column , but this method had no success. We therefore decided recently to apply centrifugation on a discontinuous sucrose gradient. However in these experiments the activity in the supernatant was considerably lower than in the previous experiments. The reason for this discrepancy is not yet known. With the gradient a separation of the detergent/protein mixture into three peaks was obtained (Fig.4).

In the upper part of this figure, the result of the sucrose gradient centrifugation of a detergent/protein mixture using a n-octylglucoside concentration of 15 mg/ml is shown. One peak that contains protein, phospholipids and ATPase activity was found around 26% sucrose. In this peak 90% of the initial protein was recovered and the phospholipid/protein ratio was 0.72 (mg/mg). This peak probably represents non-solubilized enzyme. Two other peaks, at 10% and 15% sucrose respectively were present. However, both are very small, without ATPase activity. They differ in the phospholipid/protein ratio, the lightest peak containing less lipid per protein (0.42 mg/mg),

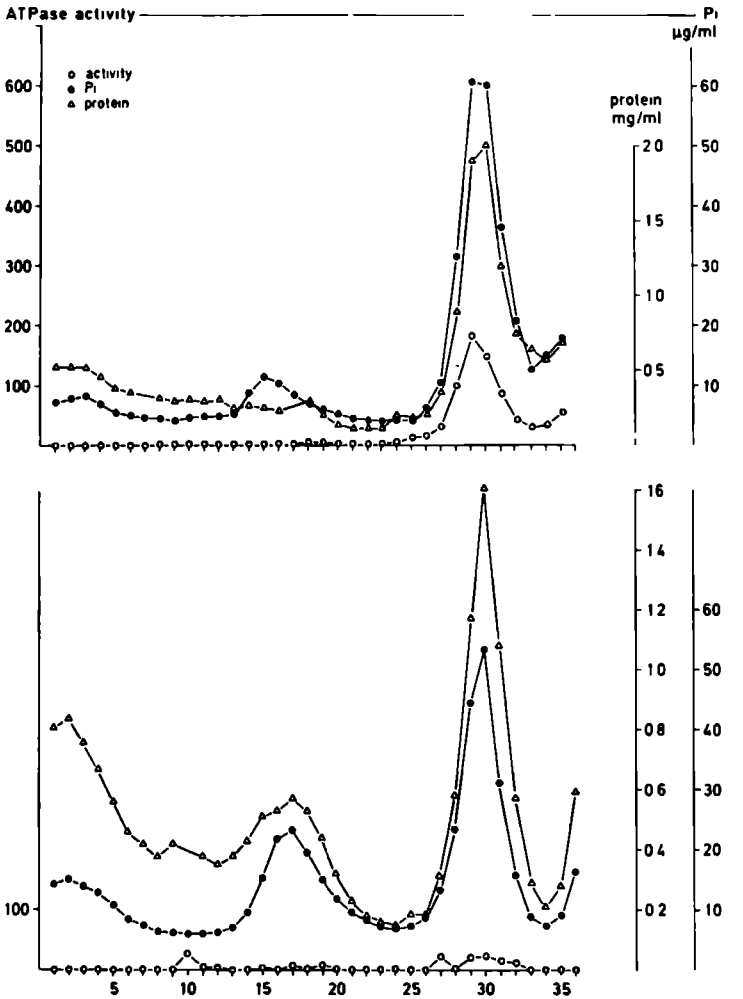

Figure 4

Sucrose gradient separation of solubilized (H^++K^+)-ATPase.

The upper figure shows the separation of the detergent/protein mixture on the sucrose gradient and the bottom figure shows the result of the centrifugation of the supernatant after centrifugation of the detergent/protein mixture for 60 min at 100,000 g.

P_i is determined in extracted lipids and the ATPase activiy is expressed in extinction units.

suggesting to be solubilized protein. In the lower part of this figure, the supernatant after centrifugation of the detergent/protein mixture in 250 mM sucrose buffer is used in the gradient centrifugation. The two lightest peaks were more prominent, compared to figure 4a, which again indicates that the first peaks were solubilized enzyme and that the third peak consisted of non-solubilized enzyme. The ratio of lipid to protein was 0.47 mg/mg in the middle peak, thus lower than the ratio described for the native enzyme (0.79) and than the ratio found in the densest peak resulting from the centrifugation of the detergent/protein mixture (0.72, fig.4a).

DISCUSSION

As described before (10), the phospholipid composition of purified preparations of $(H^+ + K^+)$-ATPase membranes is characteristic for a plasma membrane fraction, as also found for $(Na^+ + K^+)$-ATPase (11). The amount of phospholipids was estimated to be 0.79 mg per mg protein. Using phospholipases, it was possible to investigate the requirement for phospholipids for the ATPase activity. It was found that phosphatidylcholine, phosphatidylserine and phosphatidylethanolamine were the most important phospholipids, and when maximal hydrolysis of phospholipids had taken place, 70 % of the enzyme activity was lost (12,13).

During solubilization, detergent is supposed to substitute for the phospholipid environment of the enzyme, and therefore, it was of interest to investigate the effect of detergent treatment on the enzyme. Addition of detergent to native gastric membranes caused a 80% loss of activity , comparable to the effect of phospholipid hydrolysis. However, the denaturation was reversible, because a 10 fold dilution restored the activity almost completely.

The inactivation of the enzyme was shown to be time dependent and evidence was given that great part of the solubilization and inactivation was a rapid process. After this fast inactivation the remaining activity is quite stabile in time.

Only a small amount of the detergent was firmly bound to the solubilized enzyme and the majority is loosely bound. Most of the detergent can be removed by simple dilution, making it possible to restore ATPase activity almost completely. The small loss of activity that remained is probably due to the bound detergent.

TABLE 1

EFFECTS OF n-OCTYLGLUCOSIDE ON $(H^+ + K^+)$-ATPase

Distribution pattern after 1 hour centrifugation at 100,000 g as described in the METHODS section (averages are given of 4 experiments, with SD)

concentration n-octylglucoside	pellet protein	sup protein	pellet activity	sup activity	total activity compared to start
0	98 %(1)	2 %(1)	100 %(1)	- (1)	= 100 %
7.5 mg/ml	78 %(9)	22 %(9)	97 %(1)	3 %(1)	62 %(8)
15.0 mg/ml	46 %(6)	54 %(6)	76 %(7)	24 %(7)	27 %(6)
30.0 mg/ml	53 %(5)	47 %(6)	28 %(16)	72 %(16)	13 %(7)

TABLE 2

KINETIC PARAMETERS OF SOLUBILIZED $(H^+ + K^+)$-ATPase

Enzyme is solubilized with n-octylglucoside, 15 mg/ml, at a detergent/protein ratio of 1, as described in the METHODS section

	native enzyme	solubilized enzyme	
activity :	v=90 µmoles/mg.h	15.3 µmoles/mg.h	(SD=7.1,n=5)
K_m for K^+ :	3 mM	3.3 mM	(SD=0.6,n=5)
K_m for ATP :	56 µM and	57 µM and	(SD=11,n=3)
	1 mM	1.1 mM	(SD=0.2,n=3)
phospholipid content :	0.79 mg/mg protein	0.47 mg/mg prot.	(SD=0.12,n=5)
phosphorylation level :	1000 pmol/mg	110 pmol/mg	(SD=1,n=2)

These results obtained with the detergent n-octylglucoside will probably be confirmed with other mild detergents which are used for the solubilization of the $(H^+ + K^+)$-ATPase. Instead of n-octylglucoside, cholate has been used to solubilize the $(H^+ + K^+)$-ATPase, because more activity was retained after the cholate solubilization and because it was a performant tool in the freeze-thaw reconstitution method as described by Rabon et al (14,15).

To draw final conclusions, however, the methods for lipid analysis after separation of the lipids from the enzyme have to be more sensitive and another separation technique has to be tried, since it is hard to distinguish firmly attached lipids and lipids that only "stick" to the enzyme. A knowledge of that would be very useful for the understanding of the exact process of solubilization and reconstitution of $(H^+ + K^+)$-ATPase.

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CHAPTER FOUR

RECONSTITUTION OF $(H^+ + K^+)$-ATPase

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Annick Soumarmon
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SUMMARY

Gastric (H^++K^+)-ATPase was solubilized with cholate and was reconstituted into artificial vesicles, composed of 40 % cholesterol and 60 % egg phosphatidylcholine. Reconstitution was carried out by a freeze-thaw technique, completed by a sonication step.

The content of the liposomes could be varied, in order to create ion gradients over the liposomal membrane. This initiated passive exchange of H^+ for K^+, which could be inhibited with specific ATPase inhibitors, like omeprazole. This indicates the incorporation of ATPase molecules in the liposomes. The interior volume of the proteoliposomes per enzyme molecule was larger than that of the native membrane vesicles. The initial rates of transport therefore lasted longer and saturation of the vesicles occurred later, which simplified the measurement of transport processes. Reconstitution can be an important tool for the study of the sidedness of (H^++K^+)-ATPase.

Properties of the solubilized enzyme were determined and compared to those of the native enzyme. The low cholate concentrations used in the reconstitution did not lead to the same amount of solubilized ATPase, as found earlier with the detergent n-octylglucoside, described in chapter 3 of this thesis. However, since the remaining activity of the enzyme was larger, cholate was chosen for the reconstitution of the ATPase.

INTRODUCTION

Lee et al (1) showed already in 1974 that isolated gastric membrane vesicles were able to transport H^+ upon addition of ATP. This preparation has been used for transport studies (2-4) and we have applied this method in our laboratory (5).

Using preparations of native membranes has many disadvantages, for example the presence of other proteins than $(H^+ + K^+)$-ATPase, and vesicles which are "broken" during purification. This may have led to an underestimation of the transport ratio. These disadvantages can be overcome by the reconstitution of $(H^+ + K^+)$-ATPase in liposomes of known composition. Before this incorporation could be performed, solubilization of the enzyme was necessary. Soumarmon et al (6) succeeded for the first time to solubilize the ATPase in its active form. They used the mild detergent n-octylglucoside and we have described the effects of this detergent on the enzyme (chapter 3).

With the solubilized $(H^+ + K^+)$-ATPase reconstitution was possible, as shown by Rabon et al (7), who incorporated the enzyme in phosphatidylcholine /cholesterol vesicles and could measure transport mediated by $(H^+ + K^+)$-ATPase by means of a fluorescent probe. In this reconstitution method, cholate was the detergent that was applied.

In this chapter a detailed description of the method is given, which is used for further studies of the enzyme mediated transport. For this reconstitution of $(H^+ + K^+)$-ATPase, we have always used cholate and therefore, we first investigated the effect of the detergent on the enzyme. We studied in particular the effect on the lipid surroundings of the protein and the pattern of solubilized enzyme on a sucrose gradient.

METHODS

Preparation of liposomes

To measure transport processes after the incorporation of a transport enzyme, it is necessary that the used liposomes are monolamellar, in order to have an optimal transport capacity of the enzyme.

In literature different methods have been described for the preparation of liposomes. Most frequently sonication of phospholipids has been used (8-11). The results however depend on the sonication time and the apparatus used,

since multilamellar vesicles are formed easily, when heating of the mixture occurs. Dialysis of a mixture of detergent and phospholipids has also been described (12,13), but again multilamellar vesicles are often the result of this method. A third method is reverse phase evaporation (14,15). This is a rapid and reproducible method, resulting in concentrated solutions of monolamellar liposomes. In this method phospholipids are dissolved in ether and mixed thoroughly with an equal volume of water. This results in the dispersion of small water particles in the ether phase, and when then evaporation under a stream of nitrogen is carried out, phospholipids are organized in a single bilayer at the interface of the two solvents. Complete evaporation of the ether leads then to the formation of monolamellar vesicles with a diameter of approximately 1 um.

In our standard procedure for the preparation of liposomes, 20 mg cholesterol and 30 mg phosphatidylcholine (Avanti Polar Lipids, solution in chloroform) were evaporated under a stream of nitrogen. Diethylether (1 ml) was added and evaporated under nitrogen. After this washing step 1 ml diethylether and 1 ml of a buffer was added. The solution was mixed thoroughly on a Vortex mixer and after 2 min a gentle stream of nitrogen was applied to evaporate the ether, while mixing was continued. When all ether had disappeared, the evaporation was stopped. The liposomes were then filtrated through a series of filters with diameters of 1.2, 0.8, 0.65 and 0.45 μm (Millipore type WP) and sonicated afterwards for 30 min in a Branson B 12 sonicator bath, to obtain a homogeneous mixture of liposomes.

Reconstitution

The method of incorporation of transport enzymes in artificial vesicles, has been developed by Racker (9,16). This method has been used for the characterization of different ion-transporting proteins. Several methods have been described, such as sonication, detergent-dialysis and freeze-thaw cycles.

Sonication of a mixture of solubilized enzyme and liposomes is one of the first methods used. However in the case of $(H^+ + K^+)$-ATPase denaturation of the enzyme occurs during the long period of sonication necessary for the incorporation of the protein in the phospholipid bilayer. Detergent-dialysis has been applied for the reconstitution of $(Na^+ + K^+)$-ATPase (17). This method consists of a simultaneous mixing of enzyme, detergent and phospholipids. The detergent is afterwards removed from the mixture by

dialysis against a detergent-free medium. This technique requires the use of a dialysable detergent such as cholate or n-octylglucoside, but eliminates detergents as Triton X-100, whose micelles are too large to pass through the pores of the dialysis bag. On the other hand the critical micellar concentration of the used detergent should not be too small, since otherwise the dialysis time would be too long. The dialysis time of ca. 24 hours is a disadvantage of this method. With this technique reconstitution of $(H^+ + K^+)$-ATPase has been possible (18). Instead of dialysis to remove the detergent, dilution has been used. With this method the detergent concentration is rapidly brought under its cmc. The low protein concentration after the dilution is a severe disadvantage. Measurement of transport processes is difficult under these conditions. This method is less successful as compared to detergent-dialysis in the percentage of reconstituted protein. Another method to remove the detergent is the filtration of the proteoliposomes over Sephadex columns (19,20), where the detergent micelles are bound to the column material.

The most simple reconstitution method is the freeze-thaw technique. Kasahara et al (21,22) developed a method in which the protein-liposomes mixture is rapidly frozen and afterwards thawed at room temperature. This method is often completed by a short sonication step. It has been applied for the successful reconstitution of $(H^+ + K^+)$-ATPase by Rabon et al (7) and we have used this method to study the transport properties of the enzyme (23-25).

The method we have applied in general for all our reconstitution experiments is as follows. 150 µl gastric microsomes (20-24 mg/ml) in 25 mM Tris-HCl (pH 7.2) were treated with 30 µl 10 % (w/v) recrystallized cholate. This preparation was then added to 1 ml of liposomes, prepared as described earlier, giving a lipid/protein ratio of 15 (on weight basis). After thorough mixing on a Vortex mixer, the preparation was frozen in liquid nitrogen, thawed at room temperature and sonicated for 2 min at output 10 in a Branson sonicator bath. Detergent was eventually removed by centrifuging the proteoliposomes over a Sephadex G 25 coarse column in a syringe for 2 min at 750 rpm. This step was repeated once. Control experiments with ^{3}H-cholate demonstrated that the final eluate contained less than 0.02 % cholate (w/v) (23).

Preparation of the membrane fractions

Gastric (H^++K^+)-ATPase was isolated from the fundic region of pig stomachs by a series of centrifugation steps as detailed elsewhere (5,23,25). After purification, an enzyme preparation was obtained with an ATPase activity of 70-90 μmoles per mg per hour and a high specificity for ATP.

Protein determination

Protein content of the purified enzyme and of the proteoliposomes was determined either by the method of Lowry et al (26) or the Coomassie blue staining, according to Bradford (27), or with the use of a fluorimeter using an excitation wavelength of 278 nm and an emission wavelength of 340 nm. Bovine serum albumin was used as a standard.

ATPase assay

K^+-ATPase activity of the enzyme was measured in 30 mM imidazole-HCl (pH 7.5), 5 mM Mg^{2+}, 0.1 mM ouabain, 20 mM KCl or 20 mM choline-chloride and 5 mM Na_2ATP. To 400 ul of this medium, 20 μl of enzyme suspension was added, after which incubation took place for 10-30 min at 37°C. Stoppping of the reaction was done by adding 3 ml of a 1:1 mixture of 9.2 % (w/v) Fe_2SO_4 in 0.66 M H_2SO_4 + 1.15 % ammoniummolybdate and 8.6 % trichloretic acid. After 30 min the extinction at 700 nm was read and compared with the extinction of a standard treated in the same way.

Lipid analysis and phosphate determination

Lipids were extracted with the method of Folch et al (28), with the modification, that dichloromethane was used as solvent instead of chloroform.

Phosphate content of membrane samples the method of Fiske and SubbaRow (29). Samples were digested with 0.2 ml concentrated H_2SO_4 /$HClO_4$ for 1 hour at 180°C. The tubes were then cooled below 50°C. In case the destruction was incomplete, 0.1 ml 30 % H_2O_2 was added to the tubes and the destruction continued for at least 15 min, untill the samples were

TABLE 1

EFFECTS OF CHOLATE ON (H^++K^+)-ATPase

Distibution pattern after 1 hour centrifugation at 100,000 g (MSE, 10x10 rotor), pellet resuspended in 25 mM Tris-HCl (pH=7.5)

concentration cholate	protein pellet	protein sup	activity pellet	activity sup	percent activity
0 mg/ml	90%	10%	75%	25%	100%
12.5	69%	31%	97%	3%	96%
15.0	64%	36%	92%	3%	42%
18.0	73%	27%	80%	20%	59%

colourless. After cooling, 4.75 ml of colour reagent was added, consisting of a freshly prepared mixture of 50 ml of a solution containing 2.60 mg $(NH_4)_6Mo_7O_{24}.4H_2O$ and 2.2 ml of a solution containing 30.1 mg $Na_2S_2O_5$ + 11 mg $Na_2S_2O_3$ + 55 mg aminonaphtalene sulphonic acid,according to Broekhuyse (30).

The contents of each tube were mixed and incubated for 20 min in a boiling water bath. After cooling with tap water, the 820 nm absorbance was measured against water. In each determination a series of standard P_i samples was included and similarly treated.

Chemicals

Cholesterol was purchased from Sigma (St Louis,MO,USA). Egg phosphatidylcholine was purchased from Avanti Polar Lipids (Birmingham,AL,USA), Sephadex G 25 coarse from Pharmacia Fine Chemicals (Uppsala,Sweden) and filters type WP from Millipore SA (Molsheim,France).

All other chemicals were from Merck (Darmstadt,FRG).

RESULTS AND DISCUSSION

Reconstitution of gastric $(H^+ + K^+)$-ATPase has been achieved recently, by the use of a freeze-thaw-sonication procedure of a mixture of phosphatidylcholine/cholesterol liposomes and solubilized enzyme (7). This incorporation of the ATPase in monolamellar vesicles provided a tool for the study of transport processes, having several advantages as compared to the native microsomes. Using solubilized purified $(H^+ + K^+)$-ATPase, interfering proteins are eliminated; the larger volume of the proteoliposomes and the lower amount of ATPase molecules per volume ensure that the transport rates are linear in time for a longer period so that saturation occurs later. The added lipids present a lipid/protein ratio under the standard reconstitution conditions (as described in the Methods section), of 2000:1 (mol:mol) (see appendix). This value is 20 times the ratio as found in native membrane preparations (31). This fact implies that the original phospholipids are only a small amount of the lipid environment of the reconstituted $(H^+ + K^+)$-ATPase.

Assuming a density of the lipids close to 1, 50 mg lipids per ml would give an interior volume of the liposomes of 370 ul per ml if all the added

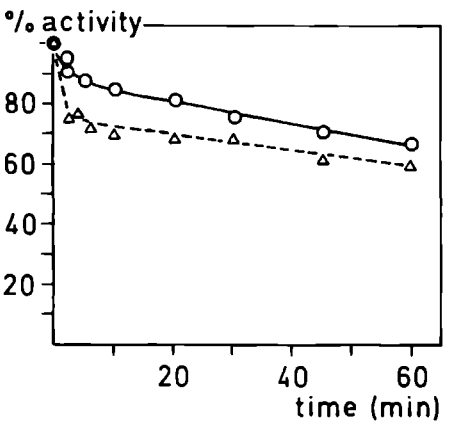

Figure 1 Time curve of inactivation of $(H^+ + K^+)$-ATPase by cholate
Concentration of protein and cholate was 13 mg/ml, incubation was on ice. The ATPase assay was carried out at 37°C for 10 min with the detergent/protein mixture (lower curve) and detergent/protein mixture which was diluted 10 times after incubation on ice (upper curve).

lipids are incorporated in liposomes (see Appendix) . This figure means that 37 % of the suspension prepared in this way would consist of liposomal space and that the transport of ions (H^+,K^+,Rb^+) can therefore be measured easily,since the interior volume is large enough to overcome saturation problems in the time period of linear transport rates. The number of vesicles per ml can also be calculated with these figures and a molar ratio of protein per vesicle can be estimated to be 2.5, when the 100 kD mass of the subunit is taken for the molecular weight of the (H^++K^+)-ATPase. For the calculation of the number of protein molecules per vesicle it is however necessary to know the amount of incorporated protein, the figure of 2.5 is the maximal one if all protein would have been incorporated.

These proteoliposomes have been used to measure the transport rates of the (H^++K^+)-ATPase, both for H^+-transport and Rb^+-transport, as described in the following chapters of this thesis. Another advantage of these proteoliposomes, compared to the native vesicles, is the possibility to vary the content of the vesicles. The choice of buffer in which the liposomes are produced can be made dependent on the kind of transport that will be measured. It is easy to create gradients of different ions in order to generate passive transport processes. This method can also be used to bring inhibitors inside the vesicles to inhibit the inside-in orientated (H^++K^+)-ATPase molecules, and to determine the the inhibitory site of agents which have effects on the ATPase activity.

Solubilization with cholate was carried out, instead of with n-octylglucoside, and this detergent was used in the reconstitution method. Since the method for reconstitution was worked out elsewhere, we did not investigate in detail the choice of the successive steps in the procedure.

We decided to investigate the effects of cholate on (H^++K^+)-ATPase and to compare them with those of n-octylglucoside, that we had used before to solubilize the ATPase and analyzed the amount of lipid in the presence of cholate (1:1). The effect of cholate as solubilizing agent was investigated in part as described for n-octylgucoside in the previous chapter . Surprisingly we found that during the first step (addition of detergent to the protein) nearly no solubilization occurs (Table 1). We rather assume that cholate molecules are incorporated in the bilayer without causing complete solubilization. When time curves of the effect of cholate on the ATPase activity were determined, it was found that the inhibition of this detergent was less than that of n-octylglucoside (fig.1). The mixture of detergent and protein was

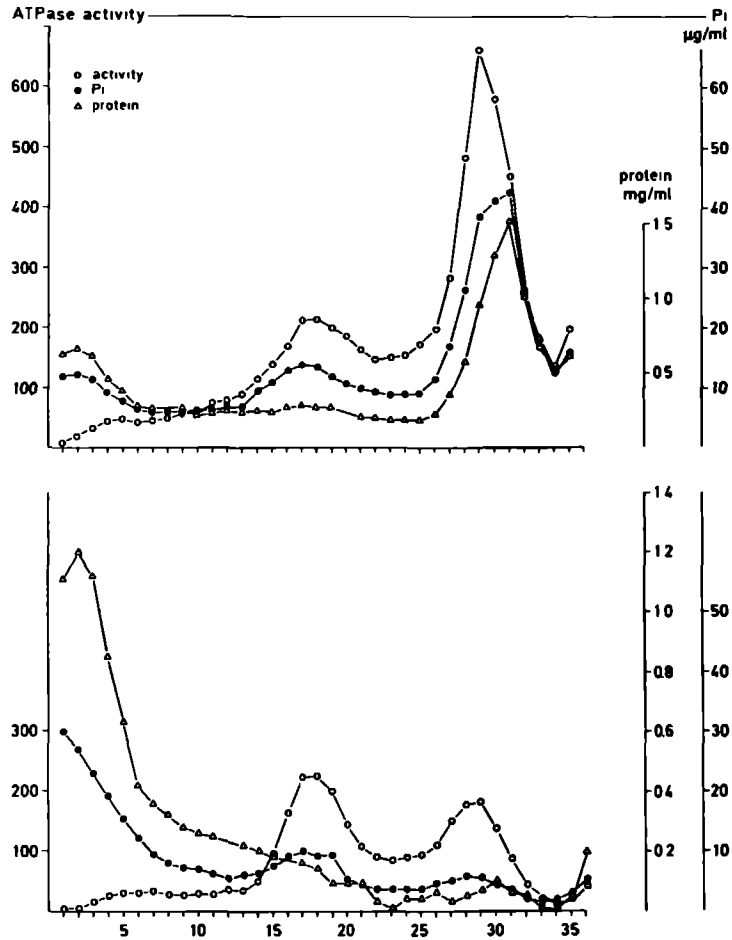

Figure 2

Sucrose gradient centrifugation of detergent/protein mixture (upper curve) and of supernatant after 1 hour centrifugation (lower curve), as described in the Methods section

P_i is determined in extracted lipids and the ATPase activity is expressed in extinction units.

brought on a sucrose gradient (fig.2). This figure shows three peaks. The peak with a density of 16% sucrose probably represents the solubilized ATPase. When only the supernatant was applied, the heaviest peak did not appear, but a very large light peak was found, that showed nearly no activity. This peak and the middle peak were assumed to consist of solubilized $(H^+ + K^+)$-ATPase. In the lightest peak a lipid/ protein ratio was found of around 0.5 mg/mg, which value was also found in the middle peak of the gradient centrifugation of the detergent/protein mixture (fig.2a). These values were lower than that of the initial ratio (0.79).

The next step in the procedure(addition of liposomes, followed by freezing, thawing and sonication) apparently leads to reconstitution as will be shown in the next chapters. Further studies, however are necessary to establish whether the various steps in the reconstitution procedure were optimal and whether the variations in the type and concentrations of lipids and detergents could improve the procedure

APPENDIX

Calculation of the liposomal volume and protein/lipid ratio

The average vesicle diameter is 2×10^{-7} nm, this means r=1×10^{-7} m.
Density of lipids is assumed to be 1 g/ml and the thickness of the bilayer membrane is assumed to be 5 nm, giving an r of 95×10^{-9} m for the internal volume. The volume of the membrane per vesicle is $4/3\pi(r_{tot}^3 - r_{int}^3)$, which is $0.6 \times 10^{-21} m^3$.
This results in a mass of the lipid membrane per vesicle of 0.6×10^{-18} kg. We started with 50 mg lipids/ml, which gives 88×10^{12} vesicles per ml. The total volume of the vesicles (internal volume + membrane) is $4.2 \times 10^{-21} m^3$ per vesicle. The volume of the vesicles per ml is then 370 µl (37 % of the total suspension).

We used 3.45 mg/ml protein under standard conditions. The content of lipid in this preparation is 2.7 mg. For the reconstitution we add 50 mg lipids, this represents 20 times the natural amount. The molar ratio lipid/protein is 100 in the purified preparation, in the reconstitution mixture therefore a ratio of 2000:1 is present.

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CHAPTER FIVE

H^+ TRANSPORT BY RECONSTITUTED GASTRIC (H^++K^+)-ATPase

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SUMMARY

Gastric $(H^+ + K^+)$-ATPase was reconstituted into artificial phosphatidylcholine/cholesterol liposomes by means of a freeze-thaw-sonication technique.

Upon addition of MgATP, active H^+-transport was observed, with a maximal rate of 2.1 umoles per mg per min, requiring the presence of 100 mM K^+ at the intravesicular site. However, in the absence of ATP a $H^+:K^+$ exchange with a maximal rate of 0.12 µmoles per mg per min was measured, which could be inhibited by the well known ATPase inhibitors vanadate and omeprazole, giving first evidence for a passive $K^+:H^+$ exchange function of gastric $(H^+ + K^+)$-ATPase.

A $Na^+:H^+$ exchange activity was also measured, which was fully inhibited by 1 mM amiloride. Simultaneous reconstitution of $Na^+:H^+$ antiport and $(H^+ + K^+)$-ATPase could explain why reconstituted ATPase appeared less cation-specific than the native enzyme (Rabon et al, 1985,J. Biol. Chem. 260,10200-10212).

INTRODUCTION

Early work by Lee et al (1) demonstrated that gastric vesicles are able to actively transport H^+ as the intact mucosa does. This property was correlated with the presence of an ATPase later called $(H^+ + K^+)$-ATPase, which actively exchanged H^+ for K^+ (2,3). The low unspecific permeability of native vesicles allowed a good characterization of ATPase transport and it was shown that in the absence of ATP, $(H^+ + K^+)$-ATPase can act as an passive $K^+ : K^+$ exchanger (4).

Reconstitution of gastric $(H^+ + K^+)$-ATPase has recently been achieved by means of freeze-thaw-sonication of a mixture of phosphatidylcholine/cholesterol liposomes and solubilized membranes (4, 5). As found with native membranes, in the presence of K^+, proteoliposomes accumulated acridine orange dye after the addition of MgATP, suggesting vesicular H^+ accumulation. Moreover, as seen with native membranes, reconstituted $(H^+ + K^+)$-ATPase also catalyzed passive exchange of K^+ in the absence of ATP (4,5). However, the rate of active H^+ transport could not be measured because of the dye technique used.

In the present paper, we used a pH electrode to measure the rate of active H^+-transport (6) and took advantage of the larger intravesicular volume to test the existence of an ATPase mediated passive $H^+ : K^+$ exchange thought to be present in native membranes.

METHODS

Preparation of the membrane fraction

Stomachs from freshly slaughtered pigs were transported to the laboratory on ice; after flushing with tap water and cleaning with paper towels, the mucosal layer was removed from the underlying tissue and homogenized in a buffer containing 250 mM sucrose and 50 mM Hepes-Tris (pH 7.2). This homogenate was centrifuged for 10 min at 2000 rpm, the pellet was homogenized once more and centrifuged again. The supernatants were pooled and centrifuged for 7 min at 25,000 rpm (Ti 70 rotor, Beckman L5-65 centrifuge), the supernatant from this step was afterwards centrifuged for 30 min at 39,000 rpm.

The resulting pellet was resuspended in the buffer and centrifuged on top

of 30 % sucrose (w/v) for 2 hours at 40,000 rpm. The opalescent 8.5 %-30 % sucrose interface was diluted twice in 50 mM Hepes-Tris (pH 7.2) and centrifuged for 30 min at 40,000 rpm. The resulting pellet which contained the $(H^+ + K^+)$-ATPase enriched membranes, was resuspended in the buffer and stored at - 30°C (4).

Preparation of liposomes

A mixture of 60 % egg phosphatidylcholine and 40 % cholesterol in chloroform was evaporated with a stream of nitrogen. After washing with diethylether, a 1:1 (v/v) mixture of diethylether and buffer was added, and the solution was thoroughly mixed on a Vortex mixer , while ether was again slowly evaporated by a stream of nitrogen.

After all ether had disappeared, liposomes were filtrated through a series of Millipore filters (WP type) from 1.2 down to 0.45 µm. Then the mixture was sonicated for 20 min in a Branson B 12 sonicator bath. The final solution contained 50 mg phospholipids per ml. The vesicles could be stored at 4°C up to one week.

Solubilization and reconstitution of the enzyme

Gastric microsomes (15-19 mg/ml), suspended in a buffer containing 40 mM Hepes (pH 7.2) were treated with 1.3 % (w/v) recrystallized cholate (final concentration). This preparation was added to a 4-5 fold larger volume of liposomes in a lipid-protein ratio of 15 (on weight basis).

After thorough mixing, the reconstitution mixture was frozen in liquid nitrogen, thawed at room temperature and sonicated for 2 min in a Branson B 12 bath sonicator. Detergent was removed from proteoliposomes by centrifuging 150 µl aliquots over a Sephadex G 25 coarse column in a 1.5 ml syringe for 2 min at 700 rpm (Diamon/IEC PR-6000 centrifuge). This step was repeated once and the final eluate was used for transport studies. Control experiments with ^{3}H-cholate demonstrated that the final eluate contained less than 0.02 % cholate (w/v).

Proton uptake measurements

The uptake of protons from the extravesicular medium was measured at 22°C by recording the change in the medium pH, with and without addition of

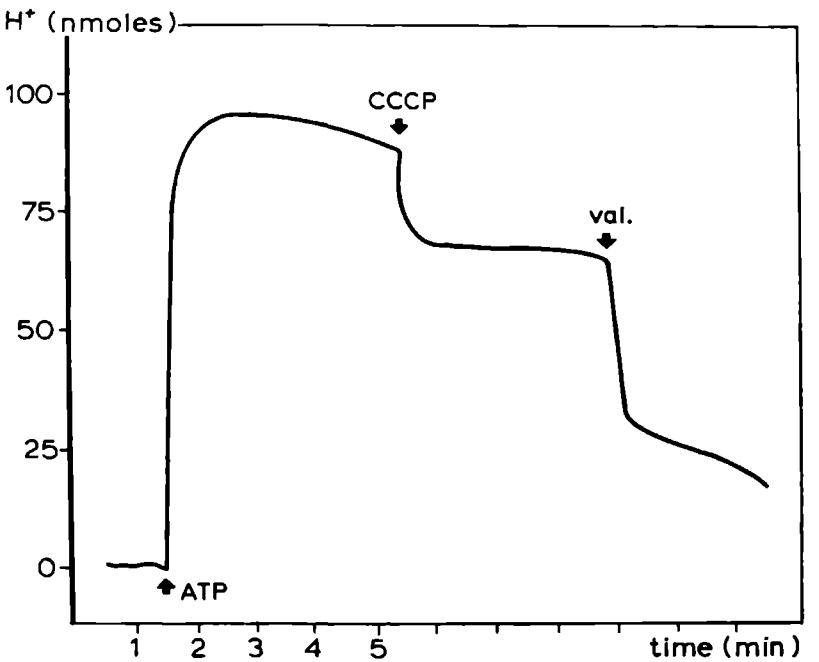

Figure 1 Active H^+-uptake by reconstituted $(H^+ + K^+)$-ATPase.
200 ul 5 mM MgATP is added to 1 ml of a suspension of reconstituted $(H^+ + K^+)$-ATPase (0.375 mg protein) in a buffer containing 5 mM glycylglycine, 2 mM $MgSO_4$ and 12.5 mM K_2SO_4 (pH=6.1). Final concentrations of CCCP and valinomycin are 25 uM and 0.1 mM resp. added as methanol solution.

MgATP.

For each experiment 150 µl of Sephadex eluted proteoliposomes were added to 850 ul glycylglycine buffer. All experiments started at pH 6.05-6.10 and the pH was continuously recorded with a Tacussel TCBC 11/HS combined electrode connected to a Tacussel ISIS 20,000 pH meter with a SEFRAM recorder. Calibration of proton uptake was done by titrating the suspension afterwards with 1 mM KOH or 1 mM HCl. Response time of the electrode was less than 1 sec.

Final concentrations of vanadate and of omeprazole were 0.5 mM and 0.2 mM, respectively. Omeprazole was prepared as a 20 mM solution in 1 mM HCl immediately before use and kept in darkness.

The ionophores CCCP and valinomycin were dissolved in ethanol and diluted to a final concentration of 25 µM. The same volume of ethanol alone had no effect.

Protein determination

Protein was determinated either by the Coomassie blue staining according to Bradford (7) or according to Lowry et al (8), using bovine serum albumin as standard.

Chemicals

Dithiothreitol, valinomycin, MgATP, CCCP (carbonyl-cyanide m-chlorophenyl-hydrazone) and cholesterol were purchased from Sigma (St Louis,MO,USA).

Egg phosphatidylcholine was purchased from Avanti Polar Lipids (Birmingham,AL,USA). Sephadex G25 coarse from Pharmacia Fine Chemicals (Uppsala,Sweden) and omeprazole was a gift from Dr B. Wallmark (Hassle AB,Sweden). Filters type WP were purchased from Millipore SA (Molsheim, France). All other chemicals were from Prolabo (Paris,France).

RESULTS

Active transport of H^+

Proteoliposomes, reconstituted by freeze-thaw sonication of a mixture of tric membranes, cholate and liposomes, took up H^+ in the presence of K^+ and in response to the addition of MgATP (fig. 1). MgATP induced H^+

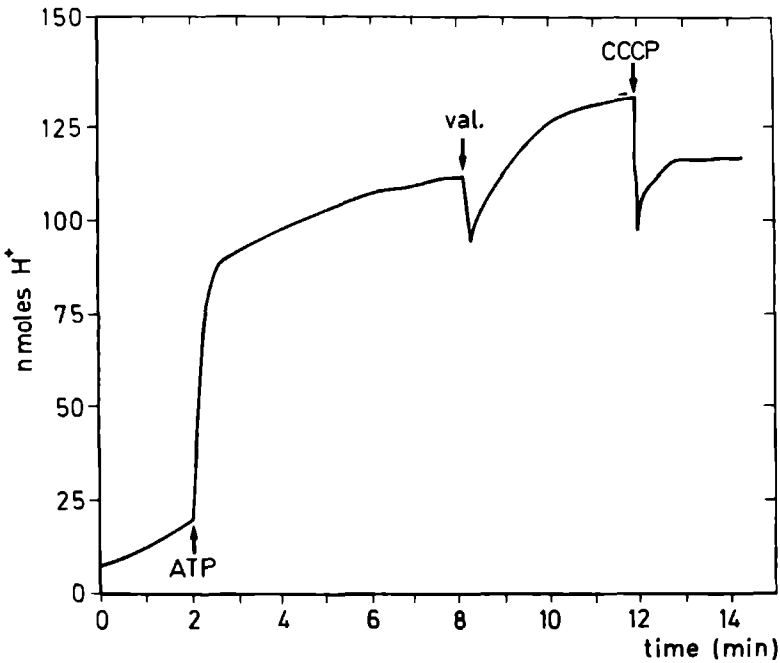

Figure 2
Active H^+-transport in the presence of a K^+ gradient.
(H^++K^+)-ATPase is reconstituted into liposomes prepared in 5 mM glycylglycine, 2 mM $MgSO_4$ and 12.5 mM K_2SO_4 (pH=6.1) and diluted 6.7 times into a buffer containing 5 mM glycylglycine, 2 mM $MgSO_4$ and 1 % sucrose (pH=6.1). Final protein concentration is 0.375 mg/ml. Initial ATP concentration is 0.25 mM and final concentrations of valinomycine and CCCP are 25 µM.

uptake was reversed by the addition of of CCCP plus valinomycin, when both intra- and extra-proteoliposomal medium contained K^+ (fig. 1). Addition of CCCP alone triggered only part of the depletion which required valinomycin to be 95-100 % completed. This was in agreement with previous results on native vesicle preparations and suggested that measured uptake mostly resulted from active exchange of H^+ for K^+ which required both H^+ (CCCP) and K^+ (valinomycin) conductances to be collapsed. Using phosphatidylcholine/ cholesterol mixtures, 10-fold higher concentrations of ionophores than used with native membranes were required. Similar concentrations had no effect on pure liposomes.

Rates of active H^+ uptake were measured in three different ionic conditions : (i) no potassium present, (ii) equilibrated potassium concentrations, (iii) potassium concentration gradient with K^+ inside proteoliposomes and no K^+ outside. Those experiments demonstrated that the highest rates of transport were measured in the presence of sulfate as anion and of an outwardly directed K^+ gradient (with sucrose in the extravesicular medium). With Na^+ present in the extravesicular medium, a lower H^+ uptake was observed, due to the existence of a Na^+:H^+ exchange, counteracting the H^+:K^+ exchange, as will be shown later.

Dilution of K^+-loaded proteoliposomes (25 mM K^+) in K^+-free medium induced a slow uptake of H^+ which was strongly accelerated by MgATP (fig. 2). When maximal uptake was reached, valinomycin could still stimulate H^+ uptake. This ionophore-dependent uptake was reversed by CCCP, suggesting that maximal H^+ uptake in the absence of ionophores was limited by the availability of intravesicular K^+.

It is to be noticed that addition of valinomycin induced a rapid drop of H^+ uptake, suggesting that a transient H^+ conductance was created. This result is not clearly understood. Addition of CCCP enhanced this drop confirming that valinomycin had created a vesicular potential outside negative by recycling K^+ into the vesicle.

Rate of active H^+ uptake depended upon the concentration of ATP used. Maximal rate was 2.1 µmoles per mg per min, in the presence of 160 mM K^+ inside and 1 mM ATP. Maximal quantity of H^+ taken up by this material (in the presence of 160 mM K^+ inside and outside the vesicles) was 270 nmoles per mg of protein, which could be compared to the 54 nmoles previously found with native membrane vesicles in the presence of the same ATP concentrations (6). This was in agreement with a larger size proteoliposomes as seen by electron microscopic examination (200 nm ± 90 nm) which could also account for

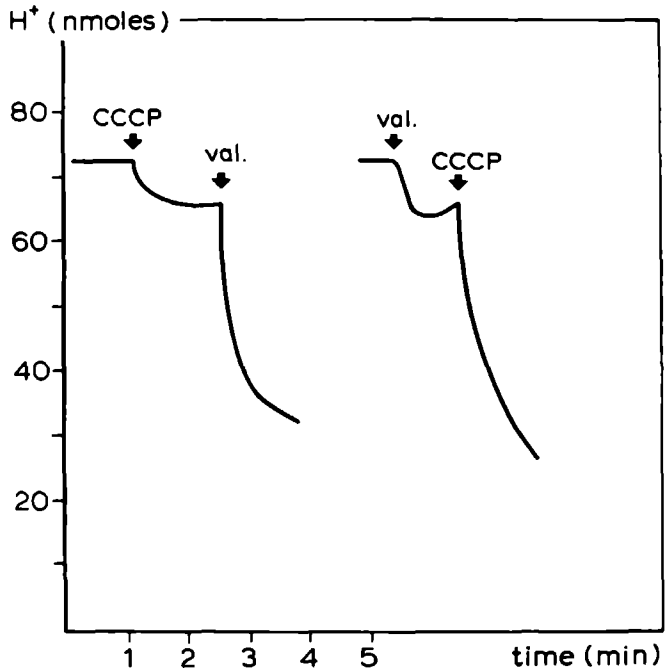

Figure 3
H^+ and K^+ conductances of PC/cholesterol proteoliposomes. $(H^+ + K^+)$-ATPase is reconstituted into liposomes prepared in 5 mM glycylglycine, 2 mM $MgSO_4$ and 1 % sucrose (pH=6.1) and diluted 6.7 0 times into a buffer containing 5 mM glycylglycine, 2 mM $MgSO_4$ and 12.5 mM K_2SO_4(pH=6.1). Final concentrations of CCCP and valinomycin are 25 µM and 0.1 mM respectively, added as methanol solutions. Final protein concentration is 0.375 mg/ml.

the slower time course of vesicle loading. Maximum uptake was reached after 70 sec in proteoliposomes and 20 sec in native vesicles.

Passive permeability

H^+ and K^+ conductances :

The passive H^+ and K^+ conductivities in proteoliposomes were determined using ionophores in the absence of ATP. In the presence of a K^+ gradient (inside 25 mM K^+/outside no K^+), addition of valinomycin created a slow H^+ leak, which was increased after CCCP addition (fig. 3). This suggests that the proteoliposomes have a low H^+ conductance. Addition of CCCP prior to valinomycin created a very small H^+ leak, which was suggestive of a low K^+ conductance (fig.3).

K^+:H^+ exchange :

Passive transport of H^+ in the absence of ionophores was found, it required the availability of a K^+ or a Na^+ counter-gradient across the proteoliposomal membrane. When K^+ or Na^+ was present inside the proteoliposomes, H^+ influx occurred, which was a H^+ efflux under opposite conditions (Na^+ or K^+ outside). The results suggested the existance of both H^+:K^+ and H^+:Na^+ exchanges. Upon increasing the K^+ counter concentration the exchange rate was saturated. The maximal rate represented approximately 5 % of the maximal active H^+ transport rate in the same preparation (fig. 4).

Effect of inhibitors

Active transport of H^+ was 80-90 % inhibited by 0.5 mM vanadate and by 0.2 mM omeprazole (ED_{50}=25 µM). Omeprazole inhibition required membranes which were free of dithiothreitol or other SH-reducing agents (fig. 5). These findings suggested that active H^+ transport was catalyzed by the (H^++K^+)-ATPase.

Passive H^+:K^+ exchange was also inhibited by 0.5 mM vanadate and by 0.2 mM omeprazole (ED_{50}=25 µM). However, these inhibitions occured only when K^+ was used as counter ion, not when Na^+ was used. By contrast, passive H^+:Na^+ exchange was inhibited by amiloride which did not affect H^+:K^+ exchange. This suggested the existance of two distinct passive transporters : the H^+:K^+ exchanger, inhibited by ATPase inhibitors and the Na^+:H^+ exchanger inhibited by amiloride.

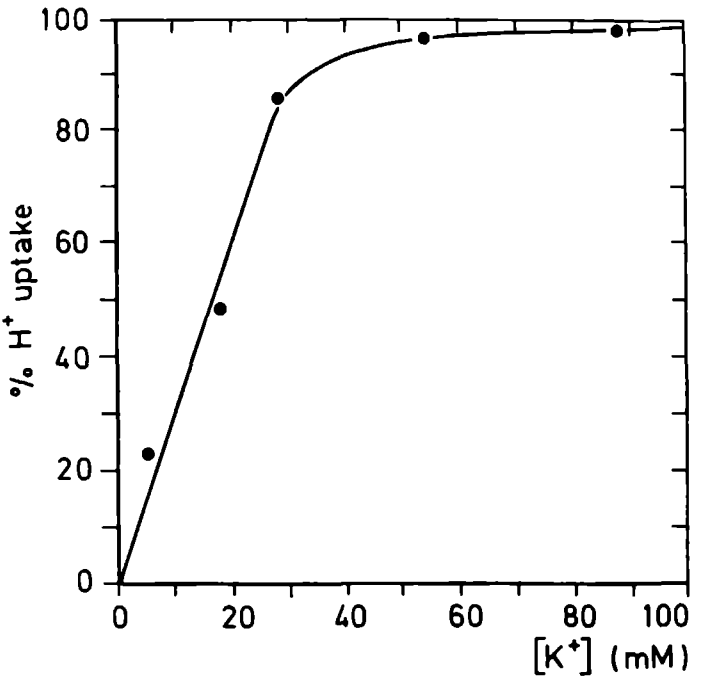

Figure 4
Saturation of passive transport by reconstituted $(H^+ + K^+)$-ATPase. $(H^+ + K^+)$-ATPase is reconstituted into liposomes prepared in 5 mM glycylglycine, 2 mM $MgSO_4$ and varying K_2SO_4 concentrations (pH=6.1) and diluted 6.7 times into a buffer containing 5 mM glycylglycine, 2 mM $MgSO_4$ and varying concentrations sucrose to maintain osmolarity (pH= 6.1). Rates of passive transport are measured at a protein concentration of 0.375 mg/ml.

DISCUSSION

It has been previously demonstrated using native gastric vesicles that the use of a pH electrode is a reliable way to quantitate H^+ transport parameters (6,9). In this paper, we used the technique to monitor H^+-transport parameters of reconstituted $(H^+ + K^+)$-ATPase. Sensitivity was high, due to the large intravesicular volume of the proteoliposomes, maximal uptake per mg of protein was 5 times that of the microsomes we have previously used (6). The relatively slow response of the electrodes (9) was less problematic because filling of vesicles lasted longer as compared to native vesicles. This is due to the dilution of the ATPase in the artificial membrane. Initial rates of passive exchanges were maintained for at least 10 minutes.

Rate of active $H^+:K^+$ exchange was dependent upon K^+ concentration and maximal rate of 2.1 µmoles per mg per min was obtained under K^+ saturating conditions. It was higher than we had previously observed with gastric microsomes (0.6 µmoles per mg per min) (6) and improvement could be partly due to the higher purity of the membrane preparation used for reconstitution as seen in specific K^+-stimulated ATPase activities of the preparation. In fact, The transport rate was closer to that estimated for gradient purified native membranes (1.4 µmoles per mg per min) (2). Gain due to reconstitution was also found in the easy availability of efficient reconstituted preparations as compared to the lability of H^+ transport function in the native fractions.

Reconstituted ATPase actively transported H^+. Higher rates of transport were measured in the presence of sulfate as compared to malonate. This should not be explained by a difference in anionic permeability but we suggest that reconstitution in the presence of malonate was less efficient.

From a previous study, reconstituted $(H^+ + K^+)$-ATPase was known to elicit active $H^+:K^+$ exchange in the presence of MgATP and a passive $K^+:K^+$ one in the absence of ATP (4). Evidence that it also catalyzed passive $K^+:H^+$ exchange, specifically inhibited by omeprazole and vanadate is new. This indicates that passive activity of $(H^+ + K^+)$-ATPase reflects all aspects of its active properties. Such a proposal was already made for $(Na^+ + K^+)$-ATPase which passively exchanges Na^+ for K^+ (10-13). Passive $(Na^+ + K^+)$-ATPase rates were 11-15 % of the maximal active ones.

It is at this point, interesting to note that passive $K^+:K^+$ exchange

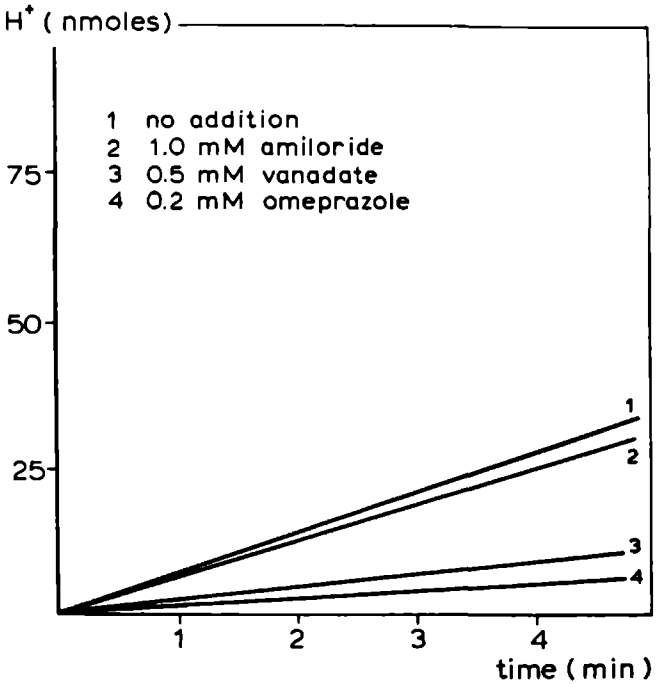

Figure 5

Passive transport by reconstituted $(H^+ + K^+)$-ATPase.

$(H^+ + K^+)$-ATPase is reconstituted into liposomes prepared in 5 mM glycylglycine, 2 mM $MgSO_4$ and 50 mM K_2SO_4 (pH=6.1). Final protein concentration is 0.375 mg/ml.

rates were very close to the maximal ATPase transport rate (5) whereas we found that passive $H^+:K^+$ exchange was only 5 % of it. This suggests that it is not the K^+-transport which is rate-limiting for the $H^+:K^+$ passive exchange capacity, but the H^+-transport.

Reconstituted (H^++K^+)-ATPase was suggested to be less specific for K^+ than the native enzyme, because rate of H^+-transport was high when intravesicular Na^+-loaded vesicles were diluted in $RbSO_4$(5). We demonstrate here that reconstitution also led to the incorporation of an amiloride sensitive $Na^+:H^+$ exchanger into the liposomes. It could account for the apparent low specificity of the ATPase, because, with Na^+-loaded vesicles, a $Na^+:H^+$ exchange coupled to a $H^+:K^+$ one, would rapidly drive K^+(or Rb^+ in the reported study) into the proteoliposomes; $H^+:K^+$ passive exchange being driven by both inside-in and inside-out reconstituted ATPase. An amiloride sensitive Na^+ transport has been previously described in gastric isolated stomachs which accounted for Na^+ apical absorption (14). Whether this transport is driven by the transporter here defined is still unclear.

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CHAPTER SIX

PASSIVE AND ACTIVE TRANSPORT BY RECONSTITUTED (H^++K^+)-ATPase

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SUMMARY

With the use of a freeze-thaw procedure, gastric $(H^+ + K^+)$-ATPase was reconstituted into artificial phosphatidylcholine/cholesterol liposomes. The reconstituted enzyme was shown to transport H^+ actively upon addition of ATP (Rabon et al, 1985, J. Biol. Chem. 260, 10200-10207). Passive H^+-transport in absence of ATP was also monitored (Skrabanja et al, 1986, Biochim. Biophys. Acta 860, 131-136). In this study we measured K^+-transport with the use of ^{86}Rb. A rate for the $Rb^+:Rb^+$ exchange of 2.8×10^{-5} mol/mg.sec was measured, being inhibited for 63% in the presence of 1 mM ATP. A model is given for the functioning of $(H^+ + K^+)$-ATPase as a passive transporter with regulatory sites for ATP, ADP and ions.

INTRODUCTION

The recent reconstitution of the gastric $(H^+ + K^+)$-ATPase into liposomes has opened new avenues for the study of the relation between enzyme structure and transport function (1). A few years ago, we described that $(H^+ + K^+)$-ATPase, which functions as an active $H^+ : K^+$ antiport in the presence of ATP, could, in the absence of this nucleotide, function as a passive K^+-antiport (2). A similar finding has been previously reported for $(Na^+ + K^+)$-ATPase, leading to a transport model, in which the acquisition of active transport capacity was triggered through regulatory sites for ATP, phosphate and Mg^{2+} (3,4). The major interest of this model is that it describes the transport process in terms of successive steps, which can be individually studied. Moreover, it allows to compare the transport cycle with the enzymatic cycle in order to investigate how the catalytic activity is involved in the transport regulation and to evaluate to what extent the passive transport refers to the pump properties. In the case of $(H^+ + K^+)$-ATPase, the existence of a passive K^+-transport was previously documented on native gastric membrane preparations. In reconstituted $(H^+ + K^+)$-ATPase passive H^+-transport was also shown to be present. The present work was carried out to further study ion transport in proteoliposomes containing reconstituted $(H^+ + K^+)$-ATPase.

METHODS

Preparation of membrane fraction

Stomachs from freshly slaughtered pigs were transported to the laboratory on ice; after flushing with tap water and cleaning with paper towels, the mucosal layer was removed from the underlying tissue and homogenized in a buffer containing 250 mM sucrose and 50 mM Hepes-Tris (pH 7.2). This homogenate was centrifuged for 10 min at 2000 rpm; the pellet was homogenized once more and centrifuged again. The supernatants were pooled and centrifuged for 7 min at 25,000 rpm (Ti 70 rotor, Beckmann L5-65 centrifuge); the supernatant from this step was afterwards centrifuged for 30 min at 39,000 rpm.

The resulting pellet was resuspended in the buffer and centrifuged on top of of 30 % sucrose (w/w) for 2 hours at 40,000 rpm. The opalescent 8.5%-30%

sucrose interface was diluted twice in 50 mM Hepes-Tris (pH 7.2) and centrifuged for 30 min at 40,000 rpm. The resulting pellet which contained the $(H^+ + K^+)$-ATPase-enriched membranes, was resuspended in the buffer and stored at -30^oC (2).

Preparation of liposomes

A mixture of 40 % cholesterol and 60 % phosphatidylcholine was evaporated under a stream of nitrogen. After washing with diethylether, a 1:1 mixture of diethylether and buffer was added and the solution was thoroughly mixed on a Vortex mixer, while ether was again slowly evaporated by a stream of nitrogen. Afterwards liposomes were sonicated for 30 min in a Branson sonicator bath (at output 10). They could be stored up to one week at 4^oC.

Solubilization and reconstitution

Gastric microsomes (20-24 mg/ml) in 25 mM Tris-HCl were treated with 1.8 % (w/v) recrystallized cholate (final concentration). This preparation was then added to a 6 fold larger volume of liposomes, giving a lipid/protein ratio of 15 (on weight basis). After thorough mixing, the preparation was frozen in liquid nitrogen, thawed at room temperature and sonicated for 2 min.

Proton uptake measurements

The uptake of protons from the extravesicular medium was measured at 22^oC by recording the change in the medium pH after addition of ATP (5). 150 µl of proteoliposomes was added to 850 µl uptake buffer, consisting of 5 mM glycylglycine, 2 mM $MgSO_4$ and 50 mM K_2SO_4 (pH 6.1). The interior volume of the proteoliposomes was either of the same composition, or K_2SO_4 was substituted by sucrose to maintain osmolarity. The pH was recorded continously with a Tacussel TCBC 11/HS combined electrode connected to a Tacussel ISIS 20.000 pH meter with a SEFRAM recorder. Calibration of proton uptake was performed by titrating the suspension afterwards with 10^{-3}M KOH or 10^{-3}M HCl. The response time of the electrode was less than 1 sec.

Omeprazole was prepared as a 20 mM solution in 1 mM HCl immediately before

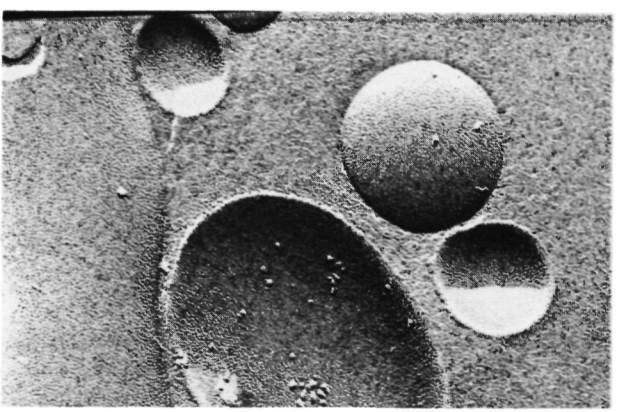

Figure 1 Freeze-fracture of proteoliposomes (x142,000)
Samples which contained no cryoprotectant or fixative were frozen at -180°C, fractured at -150°C using a Reichert-Jung cryofract 190 according to J. Escaig and covered with platinum and carbon.

use and kept in darkness. The ionophores CCCP and valinomycin were dissolved in ethanol and diluted to a final concentration of 25 μM. The same amount of ethanol alone had no effect.

Rb^+ transport measurements

Transport was measured as described previously for native gastric vesicles (2), by incubation of proteoliposomes at 30°C in the presence of tracer ^{86}Rb with or without MgATP, for determination of active and passive transport respectively.

Reaction was stopped by adding 100 μl samples to Dowex columns (6). Proteoliposomes were then eluted with 1 ml of a solution containing 250 mM sucrose and 25 mg/ml bovine serum albumin. The eluate was counted by liquid scintillation analysis.

Protein measurements

Protein was determined by the Coomassie blue staining according to Bradford (7), using bovine serum albumin as standard.

Dowex columns

Commercially available Dowex was converted from the hydrogen form to its Tris form. Columns for the assay were packed in Pasteur pipettes, up to a height of 2 cm (8).

Chemicals

MgATP, cholesterol and Dowex 50 WX 4 (200-400 mesh) were purchased from Sigma (St Louis,Mo,USA). Egg phosphatidylcholine was purchased from Avanti Polar Lipids (Birmingham,AL,USA).

^{86}Rb was from Amersham (Amersham,UK). All other chemicals were from Merck (Darmstadt,FRG). Omeprazole was a gift from Dr B. Wallmark (Hassle AB,Sweden).

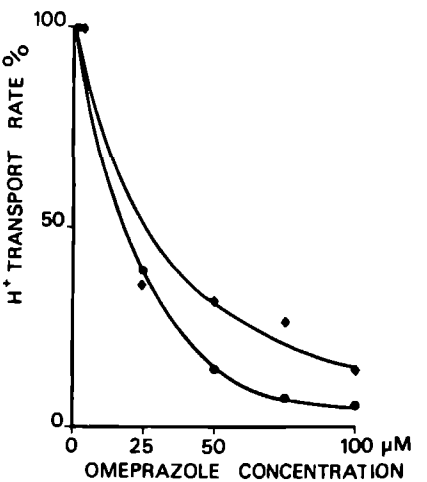

Figure 2 Inhibition of H^+-transport by omeprazole

Proteoliposomes were prepared as detailed by Skrabanja et al (12). Omeprazole was activated 10 min at pH 3 before its addition to the proteoliposome/cholate mixture. It was allowed to stay with the proteoliposomes for 30 min and then cholate was discarded by two successive centrifugations on Sephadex G25 coarse. The last centrifugation was also defined to start passive H^+ movement by generating the K^+ concentration gradient. Controls were treated the same way. Active H^+ transport rate was measured in the presence of 150 mM K^+ and 1 mM ATP. Passive H^+-transport rate was determined in the presence of 150 mM K^+ plus 20 mM glycylglycine in the proteoliposomes and sucrose plus 5 mM glycylglycine in the extravesicular medium.

RESULTS AND DISCUSSION

Gastric (H^++K^+)-ATPase membranes were solubilized by cholate (1-1.5%) and mixed with phosphatidylcholine/cholesterol liposomes as described by Rabon et al (1). The incorporation of solubilized proteins in the phospholipid bilayer can be seen in electron microphotographs of freeze-fractured proteoliposomes (fig.1). Comparison with freeze-fractured tubulo-vesicles (9) indicates that reconstitution results in dilution of the hydrophobic proteins in the phospholipid bilayer. SDS electrophoresis demonstrated that the main incorporated protein was the 95 kD subunit (1).

Quenching of acridine orange is a fast and and easy method to check the reconstitution of the ATPase by measuring the accumulation of protons (10). The response to MgATP observed with this dye on the proteoliposomes was much slower ($t_{1/2}$=2-3 min) than that reported for native membrane vesicles (11). In agreement with the feeze-fracture data, this finding could account for a larger intravesicular volume per ATPase protein. However, the tremendous amplitude of the dye response (90 to 95% quenching of the fluorescence) suggests that besides the pH dependent quenching of the intravesicular dye, large uptake of acridine orange also occurred which could be slower than the H^+-transport itself.

Further measurement of extravesicular medium pH with a sensitive electrode enabled to calculate the amplitude of the intravesicular acidification due to MgATP addition. As seen with acridine orange, alkalinization only occurred in the presence of K^+ and addition of nigericin reset the medium pH to its initial value. The pH kinetic response was faster ($t_{1/2}$=20 sec) than the acridine orange response ($t_{1/2}$=2-3 min). Maximal rate of active H^+-transport was 2.1 µmoles H^+ per mg and per min (12). The accumulation of H^+ was inhibited by vanadate and omeprazole (fig.2).

As already observed for the native preparations, reconstituted (H^++K^+) -ATPase catalyzed the passive exchange of K^+ for K^+ (or Rb^+ for Rb^+) across the membrane in the absence of ATP. As the active H^+:K^+ exchange, the passive exchange was inhibited by vanadate and omeprazole. Titration of vanadate inhibition by adding vanadate at the same time as exchange was started, suggested the presence of two sites, distinct in their apparent affinity to the anion, plus a vanadate(but not MgATP) -insensitive transport. The latter represented 27 % of total rate of $^{86}Rb^+$-uptake and was assumed to be the liposomal leak (fig.3). As vanadate inhibition was reported to be due to the high affinity vanadate site only, the existence of two equally

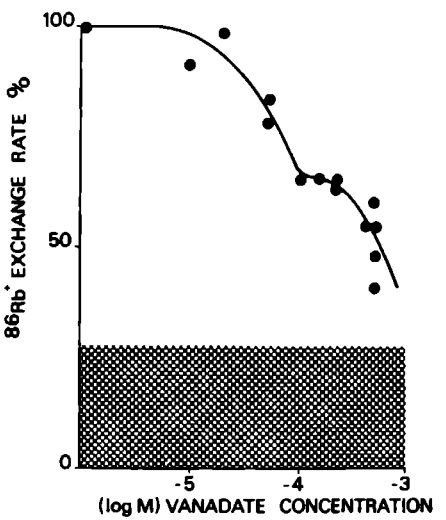

Figure 3 Inhibition of passive Rb^{+}-exchange by vanadate
Proteoliposomes were prepared as detailed by Skrabanja et al (12). Elimination of cholate by Sephadex G25 coarse centrifugation was used to induce a 150 (inside) to 2 (outside) mM K^{+} gradient at zero time. Vanadate was added at zero time and rate of H^{+}-transport was measured with a pH electrode. This plot is representative of three separate experiments. The dashed area shows the mean residual K^{+} uptake in the presence of MgATP.

represented vanadate-sensitive sites was interpretated as demonstrating a difference in site accessibility, suggesting that 50% of the ATPase molecules were reconstitituted with their cytoplasmic site in, an 50% with their cytoplasmic site outside. The Rb^+:Rb^+ exchange rate was calculated as the total $^{86}Rb^+$ transport minus the transport insensitive to 500 uM vanadate. The rate constant for this exchange was $2.8\pm0.2 \times 10^{-5}$ mol/mg.sec (n=8) i.e. close to the rate constant previously reported for native membrane vesicles (3×10^{-5} mol/mg.sec) (2). In the absence of Mg^{2+}, $^{86}Rb^+$ influx rate catalyzed by the ATPase was 63 ±9% inhibited by 1 mM ATP. This may support ATP inhibition of the K^+-exchange influx by the cytoplasmic side-out ATPases, ATP being inefficient on the cytoplasmic side-in ATPases. In the presence of Mg^{2+}, the active transport induced by ATP further depleted the proteoliposomes from their Rb^+.

In the absence of ATP, dilution of K^+-loaded proteoliposomes in K^+-free medium induced an uptake of protons (12). Conversely, dilution of K^+-free proteoliposomes in K^+-medium induced proton efflux. Thus, proteoliposomes were able to transport H^+ as long as a counter-gradient of K^+ was imposed. As evidence has been given that proteoliposomes have a low conductance for protons (12), this suggests the existence of an antiport for K^+ and H^+. This antiport was insensitive to amiloride and bumetamide (not shown) but sensitive to vanadate and omeprazole. Figure 2 shows that the sensitivities to omeprazole of the passive and of the active H^+-transport were similar both having an ED_{50} of less than 25 μM.

The reconstituted (H^++K^+)-pump has been previously suggested to be less specific for K^+ than the native ATPase and, under definite conditions, Na^+ was reported to substitute for K^+ (1). Dilution of Na^+-loaded proteoliposomes in Na^+-free medium also induced a proton uptake. However, this Na^+:H^+ exchange was neither inhibited by vanadate, nor by omeprazole, suggesting that the (H^++K^+)-ATPase was not involved, while it was fully inhibited by amiloride (Table 1). To investigate whether a Na^+:H^+ antiport could be reconstituted on the same liposome as the (H^++K^+)-ATPase and could be responsible for the apparant decrease of the ionic selectivity of the ATPase (by replacing Na^+ for K^+ through Na^+:H^+ and K^+:H^+ coupled exchanges), proteoliposomes were induced to accumulate H^+ in response to MgATP and acridine orange quenching was measured. At the nadir, 200 μM vanadate or CDTA were added to inhibit the ATPase activity of (H^++K^+)-ATPase units localized with the ATPase site outside. Under such conditions, the presence of Na^+ promoted the rapid dissipation of the

TABLE I

Proteoliposomes were prepared as described by Skrabanja et al (12). Intravesicular medium contained 20 mM glycylglycine. At zero time proteoliposomes were diluted in 25 mM Na^+ medium (sulfate). The rate of H^+ efflux wase measured at 23 ^oC in the presence of 0.35 mg of protein per ml. Inhibitors were amiloride 1 mM, omeprazole 100 uM and vanadate 200 uM.

	initial pH	inhibitor	rate of H^+-efflux $\mu moles\ mg^{-1} min^{-1}$
exp. 1	6.1	none	0.34
	6.1	vanadate	0.34
	6.1	amiloride	0.04
exp. 2	7.0	none	0.30
	7.0	vanadate	0.33
	7.0	omeprazole	0.37
	7.0	amiloride	0.06

accumulated protons. The $Na^+:H^+$ exchange was faster than the passive $H^+:K^+$ transport catalysed by the ATPase units which were localized with the ATP site inside and thus not inhibited by vanadate. This Na^+-dependent leak was inhibited by amiloride. Thus, it appears likely that reconstitution created an artificial coupling between a $Na^+:H^+$ transporter and the $(H^+{+}K^+)$-ATPase which should not exist in vivo.

From these findings the hypothesis emerges that $(H^+{+}K^+)$-ATPase could be built as a passive transporter carrying regulatory sites for ATP, ADP and ions. In the absence of ATP, passive $K^+:K^+$ exchange would be almost as fast as the active $H^+:K^+$ exchange can be (1), suggesting that no limitation in the rate of K^+-transport occurs. After ATP binding the passive $K^+:K^+$ exchange would be inhibited at the benefit of a passive $H^+:K^+$ exchange. That this latter exchange is slower than the others (12), suggests that, in the absence of Mg^{2+} the non-phosphorylated ATPase is restricted in its H^+-transport rate capacity,possibly through an occluded proton-form. Further addition of Mg^{2+}, inducing the hydrolysis of ATP and the phosphorylation of the enzyme, would then destabilize the proton to restore the full exchange rate capacity.

The authors acknowledge Dr J.C. Escaig for his essential help in the fracture of the proteoliposomes.

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CHAPTER SEVEN

TRANSPORT RATIOS OF RECONSTITUTED (H^++K^+)-ATPase

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submitted for publication

SUMMARY

Gastric $(H^+ + K^+)$-ATPase was reconstituted into artificial phosphatidylcholine/cholesterol vesicles by means of a freeze-thaw- sonication procedure.

The passive and active transport mediated by these vesicles were measured (Skrabanja,Asty,Soumarmon,De Pont and Lewin, 1986, Biochim. Biophys. Acta 860, 131-136). To determine real initial velocities, the proteoliposomes were separated from non-incorporated enzyme, by means of centrifugation on a sucrose gradient.

The purified proteoliposomes were used to measure active H^+ and Rb^+ transport, giving at room temperature velocities of 46.3 µmoles per mg per h and 42.5 µmoles per mg per h, respectively. A transport ratio of two cations per ATP hydrolyzed was also measured. These figures indicate that the enzyme catalyzes an electroneutral H^+:Rb^+ exchange.

INTRODUCTION

The enzyme $(H^+ + K^+)$-ATPase, present in the tubulovesicular system in the gastric mucosa, catalyzes the exchange of H^+ for K^+ upon hydrolysis of ATP and is involved in gastric acid secretion (1). Most studies favour an electroneutral transport of the cations (2,3,4).

One of the unsolved problems in the study of $(H^+ + K^+)$-ATPase is the ratio between the ions transported and the ATP hydrolyzed. In the past ratios of 4,2 and 1 have been postulated (5,6,7). In a previous study with isolated native gastric membrane microsomes, we found that the ratio of H^+ transported per ATP hydrolyzed was dependent on the ATP concentration and approached a value of 2 at infinite ATP concentration. In addition, the kinetics indicated positive cooperativity between the transported H^+ ions (8).

Disadvantages of the latter preparation were the presence of other proteins than the ATPase in the microsomal membrane and the presence of "broken" vesicles, both having an effect on the above measured ratio. Another limitation was the small intravesicular volume of the microsomes, so that measurement of initial transport rates was difficult, since saturation occurred rapidly.

Recently the purified gastric $(H^+ + K^+)$-ATPase has been reconstituted in phosphatidylcholine/cholesterol liposomes, using a freeze-thaw-sonication technique (9,10). These proteoliposomes accumulated H^+ upon addition of ATP, when K^+ was present intravesicularly. The rate of H^+ accumulation was higher when an outward-directed K^+ gradient was present, than with inside and outside equilibrated K^+ concentrations. The rate increased with increasing K^+ gradient, untill saturation was reached. The enzyme also catalysed a passive H^+ transport, the magnitude of which was 5 % of the active one.

In the present study, we have measured the H^+ and Rb^+ transport ratios at various ATP concentrations to establish the transport ratios. In order to do this, it was necessary to separate the incorporated enzyme from non-incorporated protein. Our measurements confirm the electroneutral behaviour of the $(H^+ + K^+)$-ATPase with the transport of 2 H^+ and Rb^+ ions per ATP hydrolyzed at high ATP concentration.

METHODS

Preparation of membrane fraction

Stomachs of freshly slaughtered pigs were transported to the laboratory on ice. After flushing with tap water and cleaning with paper towels, the mucosa of the fundic region was scraped off and homogenized in a buffer containing 150 mM sucrose, 0.2 mM EDTA and 5 mM Tris-HCl (pH 7.2). After three up-down strokes of the rotating pestle (1000 rev/min) of a Braun teflon-glass homogenizer, the mixture was centrifuged for 20 min at 20,000 g (Sorvall,GSA rotor). The resulting supernatant was centrifuged for 45 min at 100,000 g (MSE,8x50 rotor) The pellet was resuspended in 25 mM Tris-HCl (pH 7.4) and centrifuged on top of a gradient consisting of 7 % Ficoll/250 mM sucrose in 25 mM Tris-HCl over 37 % sucrose in 25 mM Tris-HCl. After 60 min centrifugation at 100,000 g (MSE,8x50), the interface was diluted in 25 mM Tris-HCl and centrifuged for 60 min at 120,000 g (MSE,10x10 rotor). The final pellet was resuspended in a small volume of 25 mM Tris-HCl and frozen at $-30^{\circ}C$. Specific ATP hydrolysis activities of these preparations were 70-90 μmoles per mg per hour.

Preparation of liposomes

A mixture of 40 % cholesterol and 60 % phosphatidylcholine was evaporated under a stream of nitrogen. After washing with diethylether, a 1 :1 mixture of diethylether and buffer (5 mM glycylglycine, 2 mM $MgSO_4$ and 50 mM K_2SO_4; pH 6.1)) was added and the solution was thoroughly mixed on a Vortex mixer, while ether was again slowly evaporated by a stream of nitrogen. Afterwards liposomes were sonicated for 30 min in a Branson sonicator bath (at output 10). They were immediateley used or were stored up to one week at $4^{\circ}C$.

Solubilization and reconstitution

Gastric microsomes (20-24 mg/ml) in 25 mM Tris-HCl were treated with 1.8 % (w/v) recrystallized cholate (final concentration). This preparation was then added to a 6 fold larger volume of liposomes, giving a lipid/protein ratio of

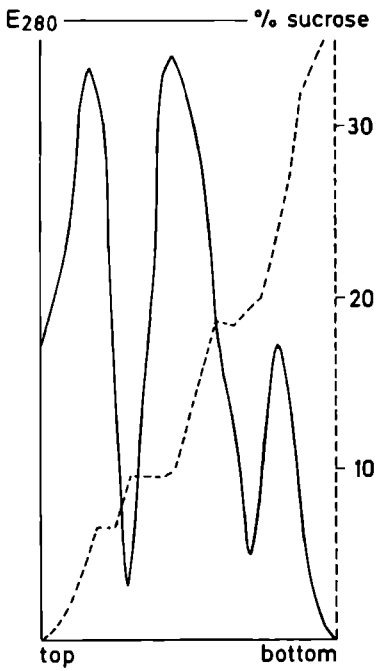

Figure 1

Absorbance spectrum of proteoliposomes centrifuged on a discontinuous sucrose gradient. The absorbance of the gradient was continuously recorded by the passage of the fractions through a Uvicord UV cell with an wavelength of absorbance of 280 nm. Details of gradient are described in the Method section.

15 (on weight basis).

After thorough mixing, the preparation was frozen in liquid nitrogen, thawed at room temperature and sonicated for 2 min at output 10 in a Branson sonicator bath.

Gradient centrifugation

The reconstituted mixture was layered on top of a discontinuous gradient of 0,10,20 and 40 % sucrose (w/v) in glycylglycine buffer (pH 6.1) and centrifuged for 90 min at 80,000 g in a TST 28-38 swing out rotor (MSE centrifuge). Afterwards fractions of 1-1.5 ml were collected from the top of the tube and the pattern at 280 nm was measured with an Uvicord UV cell.

Proton uptake measurement

The uptake of protons from the extravesicular medium was measured at 22°C by recording the change in the medium pH after addition of ATP (5). 150 µl of gradient purified proteoliposomes was added to 850 µl uptake buffer, consisting of 5 mM glycylglycine, 2 mM $MgSO_4$ and 50 mM K_2SO_4(pH 6.1). The interior volume of the proteoliposomes was of the same composition.

MgATP was added to the suspension, while the pH was continuously recorded with a Radiometer GK 2231 C combined electrode, connected with a Radiometer PHM 75 Research pH meter with a BD 40 recorder (Kipp and Sons, Delft, The Netherlands).

Calibration of proton uptake measurements was performed by titrating the suspension with known volumes of 1 mM HCl.

ATP hydrolysis measurements

50 µl aliquots of purified proteoliposomes were added to 150 µl of a medium containing 95 mM Tris-HCl, 16 mM $MgSO_4$, 0.3 mM ouabain, 0.3 mM EDTA and 32 mM choline-chloride or 16 mM K_2SO_4. 200 ul of a stock solution containing 0.87 mM ATP (for maximal ATP hydrolysis, otherwise less stock solution) and tracer γ^{32}P-ATP was then added and the mixture was incubated at room temperature.

The reaction was terminated by adding 800 µl 5 % TCA/10 % Norit (w/v) (Norit binds the unhydrolyzed ATP). After 10 min the mixture was vortexed

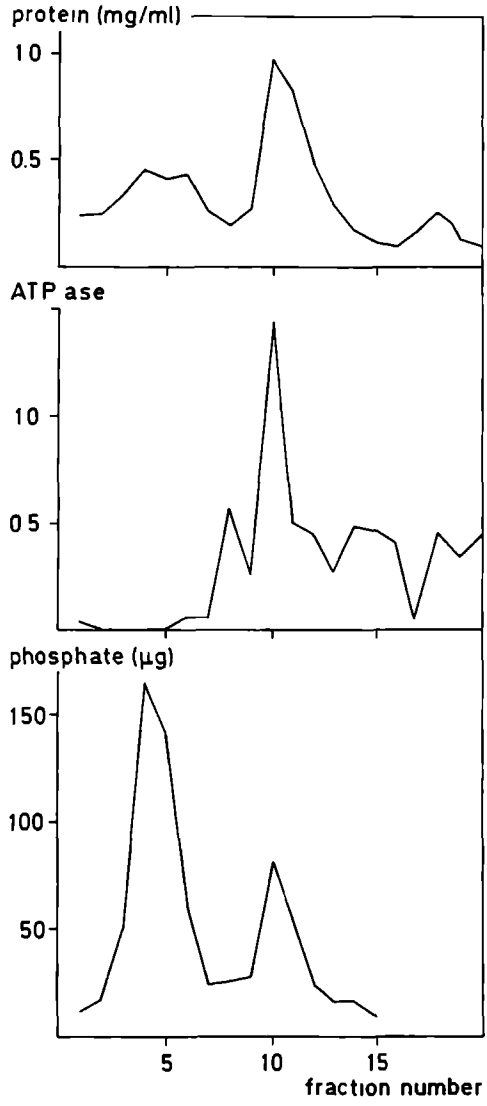

Figure 2

Sucrose gradient as shown in figure 1. The protein content (mg/ml)of the fractions was measured fluorometrically (a), ATPase activity (b) was determined as described in the Method section and phosphate content was determined after heat destruction of the fractions (c), according to Fiske and SubbaRow. Fractions of 1.5 ml were collected from the gradient, and for the above determinations 100 µl samples were used..

again, and centrifuged for 10 min at 5000 g (Hereaus Christ). 500 µl of the supernatant was taken and counted in a Philips Liquid Scintillation Analyser.

Rb^+ transport measurement

Transport of Rb^+ was measured by adding 400 µl of purified proteoliposomes to 100 µl of a buffer, containing 120 mM Tris-HCl, 500 mM Rb_2SO_4 and tracer ^{86}Rb. After 2 hours loading at room temperature, the mixture was incubated with 25 µl of a buffer containing 83.3 mM Tris-HCl, 41.6 mM $MgSO_4$ and 41.6 mM ATP (for maximal initial transport, otherwise less ATP, control was without ATP), for 30 seconds at room temperature.

Reaction was stopped by adding 100 µl samples to Dowex columns (11). Proteoliposomes were then eluted with 1 ml of a solution of 250 mM sucrose and 25 mg/ml bovine serum albumin. The eluate was counted in a Philips Liquid Scintillation Analyser by measuring Cerenkov radiation.

Protein measurement

Protein was determined with the method of Lowry et al (12) or with the use of a fluorimeter with an excitation wavelength of 278 nm and an emission wavelength of 340 nm . Bovine serum albumin was used as standard.

Lipid phosphorous measurement

Phosphate content of the gradient was determined with the method of Fiske and SubbaRow (13). Samples of 100 µl were digested with 0.2 ml concentrated $H_2SO_4/HClO_4$ for 1 hour at 180 ^oC. The tubes were then cooled below 50^oC. In case the destruction was incomplete, 0.1 ml 30 % H_2O_2 was added to the tubes, and the destruction continued for at least 15 min, untill the samples were colourless. After cooling, 4.75 ml of a freshly prepared mixture of 50 ml of a solution containing 2.60 g $(NH_4)_6Mo_7O_{24}.4H_2O$ and 2.2 ml of a solution containing 30.1 mg $Na_2S_2O_5$ + 11 mg $Na_2S_2O_3$ + 55 mg aminonaphtalene sulphonic acid (14) was added.

The contents of each tube were mixed and incubated for 20 min in a boiling water bath. After cooling with tap water and standing for 30 min, the 820 nm

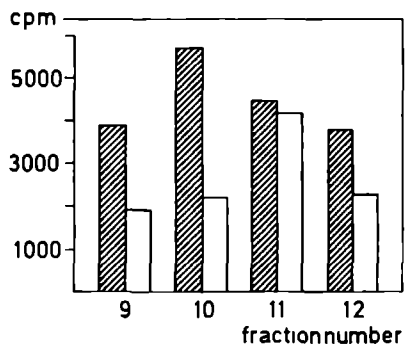

Figure 3

Fractions from gradient shown in figure 1.

Rubidium extrusion from proteoliposomes initiated by MgATP during first 30 sec of incubation at 22°C. Black bars represent the amount of ^{86}Rb taken up by the proteoliposomes passively, white represent the amount of ^{86}Rb left in the proteoliposomes after the incubation with MgATP. All other fractions showed no accumulation of ^{86}Rb. Incubation circumstances were according to the Method section.

absorbance was measured against water. In each determination, a series of standard P_i samples was incubated and similarly treated.

Dowex columns

Commercially available Dowex was converted from the hydrogen form to the Tris form. Columns for the assay were packed in Pasteur pipettes up to a height of 2 cm (15). Before use, they were treated with 1.5 ml of a solution of 250 mM sucrose and 25 mg/ml bovine serum albumin.

Chemicals

MgATP, cholesterol and Dowex 50 WX 4 (200-400 mesh) were purchased from Sigma (St Louis,MO,USA). Egg phosphatidylcholine was purchased from Avanti Polar Lipids (Birmingham,AL,USA).

γ^{32}P-ATP and ^{86}Rb were from Amersham (UK) and all other chemicals were from Merck (Darmstadt,FRG), except Ficoll from Pharmacia Fine Chemicals (Uppsala,Sweden) and sucrose from Janssen (Beerse,Belgium).

RESULTS

Gradient purification of proteoliposomes

As shown in fig. 1, a separation of the reconstitution mixture in three bands was obtained after 90 min of centrifugation. The three bands centrifuged at densities of 1.014, 1.039 and 1.094 respectively. Although after 90 min no equilibration was reached, this centrifugation time was applied because after longer centrifugation times, diffusion of the separate bands occurred.

All fractions of the gradient were collected and tested for their ability to hydrolyse ATP and to transport H^+ and Rb^+. A typical pattern of a gradient is shown in fig. 2 and fig. 3. The first (lightest) band showed nearly no ATPase activity and no transport of H^+ or Rb^+. Both other bands possessed ATPase activity, whereas the middle band had a transport capacity, both for Rb^+ (fig. 3) and H^+. Measurement of the protein concentrations by means of a sensitive fluorimetric method, yielded only small amounts in the

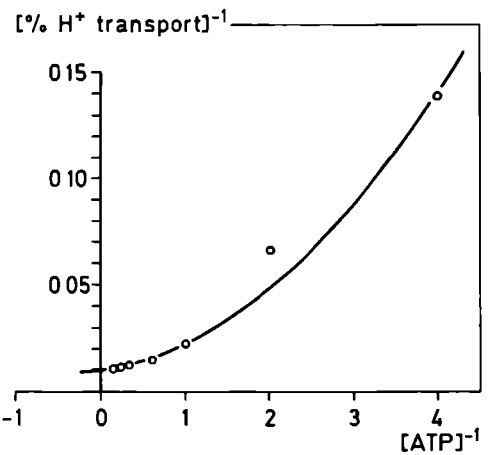

Figure 4

Lineweaver-Burk plot of proton transport versus MgATP concentration in the extravesicular medium. ATPase activity was measured as described in the Method section and H^+-transport was measured with the use of a pH electrode. Initial transport velocities during first 10 sec were calculated and plotted against the average amount of ATP in this period. This figure is typical for 5 experiments.

lightest band, indicating that this fraction contained liposomes without protein incorporated and that the lipids were responsible for the absorption peak at 280 nm (fig. 1). Inorganic phosphate, determined after complete destruction of the fractions, was only found in the two bands with the lowest density (fig. 2) suggesting that liposomes were only present in these bands. The peak with the highest density probably contained non-incorporated enzyme. In the further part of this study, only the middle band was used, since it contained the proteoliposomes.

The fact that in the third (heaviest) band no phosphate is detected, indicates only that the amount of enzyme is too small to measure the lipid phosphorous originating from the enzyme.

ATP hydrolysis

Initial velocities were measured in the first minute of incubation with ATP. The highest value obtained in the presence of 5 mM ATP was 22.0 µmoles per mg per h (SD=2.6,n=6). In the presence of 0.1 % cholate (w/v), this figure was nearly doubled, reaching 37.1 µmoles per mg per h (SD=5.0,n=4).

This indicates that in the middle peak 40 % of the enzyme molecules are rightside-in and 60 % rightside-out localized. Upon addition of ATP only the latter molecules transport H^+ and Rb^+. Bearing in mind that the activities were measured at 22°C, these values suggest higher activities than found for native purified enzyme, with known activities of 70-90 µmoles per mg per h (17), since at 37°C, ATPase activity is about 3 times higher than at 22°C (for purified membrane fraction).

H^+ and Rb^+ transport

At room temperature a maximal value for proton transport of 46.3 µmoles H^+ per mg per h (SD=5.6,n=5) and for rubidium transport of 42.5 µmoles per mg per h (SD=1.6,n=3) was found, both in the presence of 5 mM ATP. These velocities are not significantly different, indicating the same transport velocities for these processes.

Both transport mechanisms could be inhibited by 0.2 mM vanadate and in the case of Rb^+, both the passive loading of the proteoliposomes during pre-incubation and the active extrusion after ATP addition were inhibited.

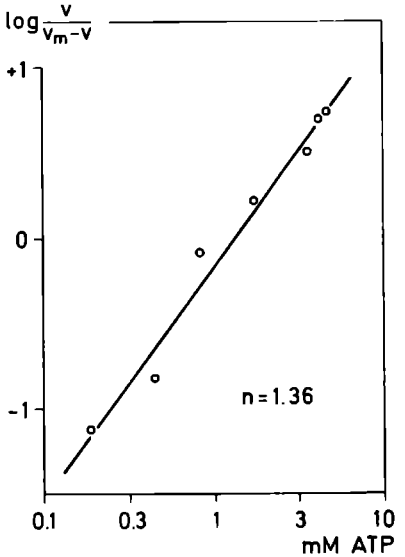

Figure 5
Hill plot of the maximal H^+ transport velocities versus the MgATP concentrations, Hill coefficient is calculated from the slope of the curve. The values obtained for the initial velocities during first 10 sec were used for this curve and plotted against the average ATP concentration during this period. This figure is typical for 5 experiments.

This inhibition was concentration-dependent, at concentrations of 0.2 mM maximal inhibition was obtained.

When detergent, i.e. cholate, was added, even low concentrations (0.1 % (w/v)), were sufficient to block completely the transport, suggesting an opening of closed vesicular systems. ATP hydrolysis was not affected by these concentrations, it was even enhanced.

Transport velocities increased at higher MgATP concentrations (fig. 4), resulting in the above mentioned maximal values. When these results were plotted in a Hill plot, an average Hill coefficient was found of 1.25 (SD=0.13,n=5). In all experiments this value was greater than 1, indicating a positive cooperativity between the two protons, as was earlier found in native gastric membrane vesicles (8). A typical example of such a Hill plot is shown in fig. 5.

From the above values, a ratio between H^+ transported and ATP hydrolyzed of 2.10 (SD=0.17) could be calculated. For Rb^+ transport, the ratio was 1.93 (SD=0.24). These values are not significantly different from 2.

DISCUSSION

In earlier studies, the proton transport capacity of native gastric membrane vesicles (8,17) and reconstituted (H^++K^+)-ATPase (9,10) has been demonstrated upon addition of MgATP. Several methods have been described to quantitate the transport velocities, such as accumulation of fluorescent dyes and of radioactive base. In our studies, we have used the pH meter method to measure H^+ transport.

Several different H^+/ATP ratios have been reported: a ratio of 1 was found by Reenstra and Forte (6), and by Smith and Scholes (7); a ratio of 2 was described by Rabon et al (5) and by ourselves (8), and even a ratio of 4 was reported by the group of Sachs (2). Reconstitution of (H^++K^+)-ATPase in artificial liposomes and the purification of these proteoliposomes gives a preparation in which "real" transport velocities can be measured, since few other proteins are present to decrease specific activities. Therefore, reconstitution can be used to settle the question of ratios. After reconstitution, purification of the proteoliposomes is necessary, since non-incorporated ATPase, capable of ATP hydrolysis, but not of transport would lead to an underestimate of the H^+/ATP and Rb^+/ATP ratios.

The proton transport velocity of 46.3 µmoles per mg of protein per h is

higher than reported previously for native membrane vesicles (8). This is presumably due to the effect of the $(H^+ + K^+)$-ATPase purification . The transport ratio of 2 protons transported per ATP hydrolyzed was, however, not affected. In parallel studies on Rb^+ transport (used as a marker for K^+ transport) we found that the maximal velocity of this transport was not significantly different from that of the H^+-transport.

These findings imply the electroneutral functioning of $(H^+ + K^+)$-ATPase; two H^+ ions would bee exchanged for two K^+ ions in each transport cycle. Earlier, electroneutrality was suggested by the absence of vesicular accumulation of lipophilic ions during ATPase activity (2,3) and by the absence of a membrane potential (4). In the present study direct evidence for the electroneutrality of the ATPase reaction is given, whereas part of the reaction cycle has recently been shown to be electrogenic (18).

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CHAPTER EIGHT

GENERAL DISCUSSION

TRANSPORT PROPERTIES OF GASTRIC (H^++K^+)-ATPase

Native membrane vesicles

Isolated gastric membrane vesicles were shown to accumulate protons upon addition of ATP (1). In different studies values were determined for the H^+/ATP transport ratio, ranging from 1 to 4 (2-5). A reinvestigation of this matter at our laboratory (chapter 2) showed that this ratio increased with increasing ATP concentration, leading at infinite ATP v concentration to a maximal value of 2. The results of other investigators could be explained by the fact that most of them took fixed ATP concentrations to measure H^+-transport, or only small variations in ATP concentrations.

The method used for proton transport measurements, a sensitive pH electrode, has advantages over the other methods described, since it is the most direct way to follow the transport of protons from the medium to the interior of the vesicles. This method is restricted to a pH of 6.1, since otherwise, the hydrolysis of ATP causes changes in the medium pH (6).

Besides the transport ratio, we found also a positive cooperativity between the two protons transported. Lineweaver-Burk plots of the transport rate versus ATP concentration were curved and a Hill coefficient significantly greater than 1 was found, indicating that the transport of one proton facilitates the transport of a second one.

The ratio of 2 protons transported per ATP hydrolyzed is thermodynamically the highest possible, when electroneutrality of the pump is assumed and a maximal pH difference generated by the vesicles of 4.5 (7). This transport ratio applies to the in vitro situation, since in vivo higher pH differences exist. Therefore the question whether only (H^++K^+)-ATPase is responsible for the acidity of the stomach can not be answered by these investigations. They imply however that the value of 4 protons per ATP is under all conditions impossible.

Reconstitution

To investigate the matter of the H^+/ATP transport ratio of further purified enzyme preparations, it was necessary to reconstitute the enzyme into phospholipid vesicles, since purification caused opening of the native

vesicles and loss of transport capacity. Before reconstitution, solubilization of the $(H^+ + K^+)$-ATPase was obligate. Several detergents have been used, with variable success, but cholate gave the best results. Low concentrations of around 2 % (w/v) gave an optimal solubilization of the enzyme and inactivation of the ATPase was minimal.

Investigations on the lipid surrounding of the enzyme after solubilization showed no dramatic effects. Only part of the phospholipids were removed by the detergent, whereas ATP hydrolysis activity was decreased during solubilization.

The choice of cholate to solubilize $(H^+ + K^+)$-ATPase was taken according the method of Rabon et al (8), who reconstituted the enzyme in phosphatidylcholine/cholesterol liposomes with success. A method of freeze-thaw-sonication was applied to incorporate the ATPase in the liposomes (chapter 4). These proteoliposomes have many advantages, as compared to native gastric membrane vesicles. The greater volume and the lower number of ATPase molecules per vesicle facilitate the measurement of transport rates, since initial velocities are maintained longer and saturation occurs later. Also the possibility to vary the interior composition of the liposomes, opens perspectives for the study of the sidedness of $(H^+ + K^+)$-ATPase.

Proteoliposomes

With the use of a sensitive pH electrode , we were able to monitor proton transport parameters of reconstituted gastric $(H^+ + K^+)$-ATPase (chapter 5). In the presence of a K^+-gradient, the ATPase mediated a passive proton transport, without ATP addition. This passive $K^+:H^+$ exchange was inhibited by vanadate and omeprazole, known inhibitors of the gastric ATPase.

This finding showed a new aspect of the functioning of the enzyme, in addition to the passive $K^+:K^+$ exchange (9). The passive $K^+:H^+$ rate was 5 % of the maximal active transport rate. In the presence of ATP, the rate was dependent upon the K^+ concentration and under saturating conditions a maximal transport rate of 2.1 μmoles per mg per min was found. This value was nearly 4 times the rate observed in native gastric vesicles (chapter 5). This could in part be due to the higher purity of the used enzyme preparation for reconstitution. That the preparation was not completely purified, was shown by the presence of an amiloride-sensitive $Na^+:H^+$ transporter. When Na^+ loaded proteoliposomes were diluted in Na^+-free medium, a proton uptake was

seen, that could not be inhibited by vanadate or omeprazole (chapter 6). This transporter could explain the previous reported fact that reconstituted $(H^+ + K^+)$-ATPase seemed to be less specific for K^+ than the native enzyme (8).

From these findings, the hypothesis can be postulated, that the enzyme is built as a passive transporter with regulatory sites for ATP, ADP and ions. In the absence of ATP, passive $K^+:K^+$ exchange is carried out by the enzyme. When ATP is bound, this exchange is inhibited and substituted by a passive $K^+:H^+$ exchange. Addition of Mg^{2+} leads to phosphorylation and active $K^+:H^+$ exchange, which is much faster than the passive one. A possible explanation might be the presence an occluded proton-form in the absence of Mg^{2+}, that would be destabilized by phosphorylation.

To determine the H^+/ATP ratio of reconstituted enzyme, it was necessary to separate the reconstitution mixture into proteoliposomes and non-incorporated protein. We applied the method of sucrose-gradient centrifugation (chapter 7). It was possible to obtain a fraction enriched with proteoliposomes, that was used for further transport measurements, in which both H^+- and Rb^+-transport rates were determined. The obtained values for both transport processes were not significantly different from each other, implying the electroneutrality of the gastric $(H^+ + K^+)$-ATPase, as was stated earlier with indirect arguments (2,10,11).

The ratio per molecule ATP hydrolyzed was not significantly different from 2, which is the same value as for native gastric membrane vesicles . This means that the influence of broken vesicles and the impurity of those preparations does not influence the transport ratio significantly and that native vesicles can be used to measure transport processes.

Future perspectives

The reconstition of gastric $(H^+ + K^+)$-ATPase into artificial lipid membranes will be of great help in the study of the sidedness of the ATPase and the study of its molecular mechanism. It is now possible to create 2 different milieus for both enzyme sides and to test ions, drugs, inhibitors and nucleotides specifically on one side of the enzyme. Tryptic digestion and specific chemical modification would also be interesting, before and after reconstitution, to compare the residual transport function of the enzyme and its ATP hydrolysis activity and the connection between them.

Another possibility would be the incorporation of subunits of the enzyme and the test of their transport capacity, to find out the minimum conditions for complete transport. Since the discussion over the hetero- or homogeneity of the subunits has not finished yet, this aspect would be of great interest.

The production of monoclonal antibodies against the enzyme is necessary to caracterize the $(H^+ + K^+)$-ATPase completely biochemically as well as histologically. When this has been done, a comparison could be made with other H^+-transporting ATPases, as found in lysosomes, endocytose vesicles, "clathrin coated" vesicles, mitochondria, to obtain insight in different modes of proton transport in nature.

Another point of interest would be the exact knowledge of the solubilization behaviour of the enzyme, the interaction between detergent and lipids and protein. In chapters 3 and 4 first steps are taken , but still, most of the effects on the ATPase are unknown.

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SUMMARY

In this thesis we describe transport functions of the Mg^{2+}-dependent, H^+-transporting, K^+-stimulated adenosine triphosphatase isolated from the gastric parietal cell, the so-called $(H^+ + K^+)$-ATPase.

We have purified native gastric membrane vesicles from the mucosa of pig stomach, in order to investigate the H^+-transport of the enzyme in relation to the ATP hydrolysis. A maximal transport ratio of 2 H^+ per ATP hydrolyzed was found, as described in chapter 2.

Using native vesicles the question of the reliability of these values could rise, since the enzyme in this preparation was only partially purified, and some of the vesicles could be leaky. Both these circumstances could result in a discrepancy between the observed (measured) and the "true" transport ratio of the gastric $(H^+ + K^+)$-ATPase. When the enzyme was further purified, the transport capacity was lost, probably since the centrifugation steps during purification damaged the vesicles. These purified preparations could therefore not be used to study transport mediated by the ATPase and another method was necessary to investigate the transport functions of the enzyme. The problem could be solved by the incorporation of purified ATPase in artificial membrane systems, phospholipid liposomes of known composition. This so-called reconstitution had been successfully used for other transport enzymes, and several techniques for this procedure have been described.

Before reconstitution the protein has to be solubilized, by the use of a detergent. The solubilization of $(H^+ + K^+)$-ATPase with n-octylglucoside is described in chapter 3 . As expexted, the detergent treatment can cause inactivation of the enzyme, and it was important to find optimal solubilization conditions, both with respect to residual activity and protein solubility. This detergent caused however a great loss of ATPase activity. Better results were obtained with cholate as shown in chapter 4 , so this detergent was then used in the reconstitution procedure. The properties of the solubilized protein are also described, and a comparison is made with the native enzyme.

The reconstitution procedure used for the $(H^+ + K^+)$-ATPase is a freeze-thaw method, followed by a short sonication. This method was first applied to the enzyme by Rabon et al (1) in 1985, and was shown to be successful to measure transport properties. This method was learned during a stay at the laboratory of Dr. Lewin and Dr. Soumarmon (INSERM U10) and the exact procedure is described in chapter 4. The methods we had used on native

gastric vesicles were applied on the reconstituted material and the H^+-transport was measured, as described in chapter 5. We were able to demonstrate that the (H^++K^+)-ATPase has the capacity to passively transport ions in the absence of ATP, in addition to the well-known active ATP driven transport. This was the first time, that passive H^+-transport was shown to exist and that fact is of great importance for the understanding of the mode of function of (H^++K^+)-ATPase. With these findings a complete scheme can be postulated with passive and active pathways, as was done for (Na^++K^+)-ATPase by Karlish and Stein (2-4).

Rubidium transport and the effect of ATPase inhibitors are described in chapter 6, and a possible explanation for the different types of transport mediated by (H^++K^+)-ATPase is given.

In chapter 7, the determination of the transport ratios of the reconstituted gastric (H^++K^+)-ATPase is described. For the determination of these ratios, it was necessary to separate the proteoliposomes from the non-incorporated enzyme. We used a sucrose-gradient centrifugation, by which it was possible to separate the reconstitution mixture in separate bands of liposomes, proteoliposomes and enzyme. The purified proteoliposomal fraction was used to determine the H^+- and the Rb^+-transport at different ATP concentrations, in analogy with the investigations done earlier with native membrane vesicles. Under these conditions both a H^+/ATP transport ratio and a Rb^+/ATP ratio of 2 were found. This implied the electroneutral exchange of H^+ for Rb^+ or K^+, a feature of the enzyme that was assumed after the results of indirect measurements (5-7), but had never been demonstrated in a direct comparison of both transport rates as in our experiments.

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SAMENVATTING

In dit proefschrift wordt onderzoek beschreven dat gedaan is naar de transportfuncties van een Mg^{2+}-afhankelijk, H^+-transporterend adenosine triphosphatase, $(H^+ + K^+)$-ATPase genoemd, uit de parietale cel van de maag.

Aan dit enzym werd al eerder onderzoek gedaan op het laboratorium voor Biochemie, maar daarbij werd hoofdzakelijk het werkingsmechanisme van het enzym bestudeerd. Het was bekend dat het enzym verantwoordelijk is voor de maagzuursecretie van de parietale cel door in een uitwisseling met kalium-ionen protonen naar het maaglumen te transporteren. Dit transport is gekoppeld aan de splitsing van ATP in ADP en anorganisch fosfaat. De vraag was nu of dit $(H^+ + K^+)$-ATPase alleen in staat is om de lage pH in de maag te handhaven en hoe de moleculaire verhouding is tussen protonentransport en de gesplitst ATP (de H^+/ATP ratio).

De mogelijkheid om protonentransport te meten werd geboden door varkensmagen te nemen, de bovenste cellaag van de maagwand af te schrapen, en deze voorzichtig te homogeniseren. De membraanstructuren uit de cellen, waarop het ATPase zich bevindt, sluiten zich dan tot zogenaamde vesikels, waarin het enzym andersom georienteerd is, vergeleken met de natuurlijke situatie (inside-out orientatie). Als er ATP beschikbaar is kan het enzym protonen naar binnen transporteren. Dit proces kan men meten met een gevoelige pH electrode als aan een oplossing met daarin deze vesikels ATP wordt toegevoegd. Door dit te doen bij verschillende hoeveelheden ATP kan men zien hoe het protonentransport varieert met de ATP concentratie. Dit wordt in hoofdstuk 2 beschreven. We vonden dat er maximaal 2 protonen getransporteerd worden per molekuul ATP dat gesplitst wordt. Ook vonden we dat deze verhouding kleiner is bij lage ATP concentraties. Bovendien hebben we laten zien dat er een cooperativiteit bestaat tussen de 2 protonen, dat wil zeggen dat het tweede proton makkelijker getransporteerd wordt door het $(H^+ + K^+)$-ATPase als er al een proton door dat enzym getransporteerd wordt.

Uitgaand van de hoeveelheid energie die vrijkomt bij het splitsen van een molekuul ATP laten we zien dat het mogelijk is om 2 protonen te transporteren tegen een concentratiegradient in, zoals die bestaat tussen de maaginhoud en de parietale cel. Dit houdt dus in dat het thermodynamisch mogelijk is voor het $(H^+ + K^+)$-ATPase om te zorgen voor de grote hoeveelheid zuur in de maag.

Het werken met deze membraanvesikels heeft wel enkele nadelen als men transportsnelheden van het protonentransport wil berekenen. Op de eerste

plaats zijn er behalve het (H^++K^+)-ATPase nog andere eiwitten aanwezig in de membranen van deze vesikels. Bij een berekening van de transportsnelheid per hoeveelheid eiwit zal men op deze manier een te lage snelheid verkrijgen. Ook bestaat de mogelijkheid dat een deel van de vesikels "lek" zijn en dus geen transport gemeten kan worden.

Daarom werd besloten verder te gaan met een zuiverder enzympreparaat. Dit wordt verkregen door de natuurlijke vesikels nog enkele keren te centrifugeren over een sucrose-dichtheidsgradient. Op deze wijze wordt een preparaat verkregen dat voor 85-90% bestaat uit (H^++K^+)-ATPase. Het nadeel hiervan is dat zo alle vesikels lek zijn gemaakt en dus ook hiermee geen transport gemeten kan worden. De mogelijkheid bestaat nu om dit eiwit in te bouwen in kunstmatige vesikels, gemaakt van fosfolipiden. In de natuurlijke situatie is het ATPase ook omgeven door fosfolipiden (de membraan is hieruit opgebouwd) en als men dan de juiste fosfolipiden neemt om er vesikels van te maken is inbouw in principe mogelijk.

In de meest gezuiverde preparaten is het enzym nog steeds omgeven door fosfolipiden (0.79 mg/mg eiwit). Voor de inbouw is het nodig om daar zoveel mogelijk van kwijt te raken en alleen eiwit over te houden. In hoofdstuk 3 wordt beschreven hoe we dit geprobeerd hebben met het detergens n-octylglucoside . Als het enzym hiermee behandeld wordt is het inderdaad mogelijk om een groot gedeelte van de aanwezige hoeveelheid enzym "op te lossen" door het van zijn fosfolipidenomgeving te ontdoen. Het blijkt echter ook dat dan de activiteit van het eiwit voor het grootste deel verloren gaat. De fosfolipidenmantel is dus nodig voor het functioneren van het ATPase. Vanwege de matige resultaten met dit detergens, hebben we voor een ander detergens gekozen, te weten cholaat. De resultaten hiervan zijn in hoofdstuk 4 beschreven. Cholaat lost minder enzym op, maar er blijft meer activiteit over en het is daarom gunstiger om dit detergens te gebruiken.

De methode om het eiwit in te bouwen in de kunstmatige vesikels (liposomen) werd in Parijs geleerd in het laboratorium van Dr. Annick Soumarmon en Dr. Miguel Lewin. Deze methode is een combinatie van reeds bekende technieken voor inbouw van eiwitten in liposomen (reconstitutie). Met cholaat wordt het eiwit eerst opgelost, daarna bij de liposomen gevoegd en in vloeibare stikstof ingevroren. Tenslotte wordt het bij kamertemperatuur ontdooid en ultrasoon getrild.

Hoofdstuk 5 beschrijft de eerste resultaten van ons gezamenlijke onderzoek naar de transportfunkties van het ingebouwde ATPase. Net als met de natuurlijke vesikels uit hoofdstuk 2 konden we protonentransport meten nadat

we ATP hadden toegevoegd aan een oplossing van de liposomen met ingebouwd eiwit (proteoliposomen). Daarnaast vonden we ook nog iets heel nieuws, namelijk dat ook zonder ATP toe te voegen, het ATPase protonen kon transporteren. Nodig daarvoor was alleen dat aan de de proteoliposomen geen kalium bevatten en de buiten-oplossing wel, of omgekeerd. Omdat het enzym protonen tegen kalium-ionen uitwisselt, ging hiermee protonentransport gepaard. Het protonentransport met en zonder ATP (actief en passief) in is dit hoofdstuk 5 uitvoerig beschreven. Hoofdstuk 6 gaat verder in op het kaliumtransport. Om dit transport te meten maakten we gebruik van een ion dat op kalium lijkt en radioactief is, namelijk rubidium. We konden dan verschillen in radioactiviteit meten om het transport te volgen. Het $(H^+ + K^+)$-ATPase maakt vrijwel geen verschil tussen rubidium en kalium en we kunnen de resultaten van het rubidium transport gebruiken om conclusies te trekken over het kalium transport.

Met deze proteoliposomen wilden we net als in hoofdstuk 2 de verhouding van transport en splitsing van ATP meten. Om dit te kunnen doen was het nodig om eerst de proteoliposomen te scheiden van eiwit dat niet ingebouwd is en van liposomen waarin geen eiwit is ingebouwd. In hoofdstuk 7 staat beschreven hoe we dat met behulp van centrifugatie in sucrose gedaan hebben, gebruik makend van het verschil in gewicht per volume (dichtheid) van de verschillende vesikels en eiwitten. Daarna hebben we de verhoudingen gemeten van protonen transport en kalium (rubidium) transport. We vinden dan dat er 2 protonen (net als in hoofdstuk 2) en 2 rubidiumionen getransporteerd worden per gesplitst ATP molekuul. Hieruit kunnen we dus concluderen dat er 1 proton tegen 1 kaliumion wordt uitgewiseld, hetgeen een electroneutrale uitwisseling betekent. Er vindt geen netto ladingtransport plaats.

Hoofdstuk 8 geeft nog een overzicht van alle resultaten en de daaruit getrokken conclusies, en als slot nog wat suggesties over wat men verder kan onderzoeken, door gebruik te maken van de in dit proefschrift beschreven reconstitutiemethode.

CURRICULUM VITAE

Ik ben geboren op 5 september 1956 in Waubach.Na 4 jaar kleuterschool heb daar zes jaar lang met tegenzin de St Joseph school bezocht. Aansluitend atheneum B gevolgd op het Eykhagencollege te Schaesberg, de laatste 2 klassen van de middelbare school bezocht ik het Bisschoppelijk College te Roermond (Broekhin).

Na het eindexamen in 1974, ben ik in Nijmegen biologie gaan studeren, met als hoofdvak microbiologie (prof Vogels) en als bijvakken exobiologie (dr Heinen) en biogeologie (dr Theunissen). Doctoraalexamen deed ik op 6 mei 1980, daarna verdween ik 14 maanden van de arbeidsmarkt en in het leger. Per 15 november 1982 kwam ik op het lab voor biochemie van de Medische Faculteit van de Katholieke Universiteit te Nijmegen werken als wetenschappelijk assistent op een projekt getiteld: "de rol van $(H^+ + K^+)$-ATPase bij zuursecretie in de maag". Behalve de onderzoektaak, had ik een onderwijstaak, die tot uiting kwam in assistentie bij het 1^e en 2^e jaars biochemie praktikum voor studenten geneeskunde en het begeleiden van twee HBO stagiaires en een scheikunde-student (Alexander,Jacintha en Riny).

Naast al deze geestelijke belommeringen, vond ik nog tijd een atletiekloopbaan op te bouwen. Voorjaar 1983 debuteerde ik op de marathon en op 26 februari 1984 liep ik een nog steeds bestaand clubrecord voor de AV Heythuysen in $2^h 42'55''$. Kort daarna werd mijn eerste artikel in Biochim. Biophys. Acta gepubliceerd, over de transport ratio van geisoleerde membraan vesikels uit de maag, zoals opgenomen is in dit proefschrift. Die zomer beklom ik per fiets o.a. de Mont Ventoux en de Galibier, tijdens een 2000 km fietstocht door de Franse Alpen. Begin 1985 verbleef ik 4 maanden in Parijs, om aan het INSERM U10 samen met Annick Soumarmon onderzoek te verrichten aan gereconstitueerd $(H^+ + K^+)$-ATPase. Dit verblijf leverde een in dit proefschrift opgenomen gezamenlijk artikel op en een clubrecord over 30 km ($1^h 49'00''$), gelopen in Acheres. Na terugkeer uit Parijs, heb ik te Nijmegen de opgedane ervaringen en technieken verder uitgewerkt in mijn eigen onderzoek, hetgeen resulteerde in de in dit boekje beschreven resultaten. In de zomer van 1986 fietste ik in de bandsporen van Coppi over de Val Gardena en de Pordoi in de Dolomieten, dit leverde de energie op om het laatste gedeelte van mijn promotieonderzoek met enthousiasme te beginnen en ook aan het proefschrift zelf.Per 1 januari 1987 is mijn werkzaamheid op de afdeling biochemie beeindigd en daarna heb ik dit proefschrift afgerond.

De H^+/ATP ratio van 4 voor het $(H^+ + K^+)$-ATPase is thermodynamisch gezien niet mogelijk en moet verklaard worden door het gebruik van slecht materiaal.

Sachs G. et al (1976) J. Biol. Chem. 251, 7690-7698

Skrabanja A.T.P. et al (1984) Biochim. Biophys. Acta 774, 91-95

De aanwezigheid van een $Na^+:H^+$ exchanger in het gebruikte enzym preparaat is een verklaring voor de schijnbaar lagere kation specificiteit van gereconstitueerd $(H^+ + K^+)$-ATPase.

Rabon E.C. et al (1985) J. Biol. Chem. 260, 10200-10207

Skrabanja A.T.P. et al (1986) Biochim. Biophys. Acta 860, 131-136

Het feit dat het $(H^+ + K^+)$-ATPase kationen zowel actief als passief kan transporteren is een verder argument voor de overeenkomst met $(Na^+ + K^+)$-ATPase.

Karlish S.J.D. and Stein W.D. (1982) J. Physiol.(London) 328,295-350

Skrabanja A.T.P. et al (1986) Biochim. Biophys. Acta 860,131-136

Methoden die gebruikt worden voor een isolatie van een $(Na^+ + K^+)$-ATPase remmer uit serum kunnen bijdragen tot het ontstaan van die remmer.

Kelly R.A., O'Hara D.S., Mitch W.E. and Smith T.W. (1986)
J. Biol. Chem. 261, 11704-11711

Bij het opsporen van de kation bindingsplaatsen op het $(Na^+ + K^+)$-ATPase molecuul verdient het de voorkeur van lipofiele kationen gebruik te maken.

Schuurmans Stekhoven F.M.A.H. (1987) unpublished results

Endopeptidases en proteinases verschillen alleen in hun historische achtergrond en niet in hun functie.

Barrett A.J. and McDonald J.K. (1986) Biochem. J., 935

Polyamines reguleren de Ca^+-afhankelijke eiwitfosforylering systemen in vivo.

Qi D.F., Schatzman R.C., Mazzei G.J., Turner R.C., Raynor R.L., Liao S. and Kuo J.F. (1983) Biochem. J. 213, 281-288

Het feit dat Peters et al vonden dat antisera tegen $(Na^+ + K^+)$-ATPase geen reactie vertonen met $(H^+ + K^+)$-ATPase kan verklaard worden door hun gebruik van ^{125}I-gelabeld protein A om antigeen gebonden antilichamen te identificeren.

Peters W.H.M., Ederveen A.G.H., Salden M.H.L., De Pont J.J.H.H.M. and Bonting S.L. (1984) J. Bioenerg. Biomemb. 16, 223-232

Lane L.K., Kirley T.L. and Ball Jr W.J. (1986)
Biochem. Biophys. Res. Commun. 138, 185-192

Uit cristallizatie experimenten valt af te leiden dat de functionele eenheid van het $(H^+ + K^+)$-ATPase bestaat uit 2 α monomeren.

Rabon E., Wilke M., Sachs G. and Zampighi G. (1986)
J. Biol. Chem., 261, 1434-1439

Het feit dat Joop Zoetemelk toch nog de Amstel Gold Race heeft gewonnen toont weer dat de aanhouder altijd wint.

Het alleen voor mannelijke promovendi gehanteerde kledingvoorschrift voor de promotie is in strijd met de gelijke behandeling van man en vrouw.

Het kleden van de scheidsrechters in Ajaxshirt zou de situatie op het speelveld duidelijker maken, gezien het voorschrift dat de scheidsrechter passende kleding dient te dragen.

Het tegen de Nijmeegse krakersbeweging gebruikte wetsartikel 140 is ook van toepassing op de regering.

Wetboek van strafrecht, art. 140

Zelfs mensen die op hun hoofd gevallen zijn kunnen promoveren.

Arno Skrabanja

Nijmegen 23 juni 1987